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TITLE: GENERATION AND SELECTION OF NOVEL BINDING PROTEINS

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GENERATION AND SELECTION OF NOVEL BINDING PROTEINS

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GENERATION AND SELECTION OF NOVEL BINDING PROTEINS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to development of novel binding proteins by an iterative process of mutagenesis, expression, chromatographic selection, and amplification.

Information Disclosure Statement

The amino acid sequence of a protein determines its three-dimensional (3D) structure, which in turn determines protein functioning (EPST63, ANFI73). A widely accepted system of classifying protein structure may be found in Schulz and Schirmer (SCHU79, Ch5). Their classification system is adopted herein.

Shortle (SHOR85), Sauer and colleagues (PAKU86, REID88), and Carutners and colleagues (ZISE85) have shown that some residues on the polypeptide chain are more important than others in determining the 3D structure of a protein. The 3D structure is essentially unaffected by the identity of the amino acids at some loci; at other loci only one or a few types of amino acid is allowed. In most cases, loci where wide variety is allowed have the amino acid side group directed toward the solvent. Loci where limited variety is allowed frequently have the side group directed toward other parts of the protein. Thus substitutions of amino acids that are exposed to solvent are less likely to affect the 3D structure than are substitutions at internal loci. (See also SCHU79, p169-171 and CREI84, p239-245, 314-315).

The secondary structure (helices, sheets, turns, loops) of a protein is determined mostly by local sequence. Certain amino acids tend to be correlated with certain secondary structures and the commonly used Chou-Fasman (CHOU74, CHOU78a, CHOU78b) rules depend on these correlations. The correlations between amino-acid type and secondary structure are not, however, absolute, and every amino acid type has been observed in helices and in both parallel and antiparallel sheets. Kabsch and Sander (KABS84) report on pentapeptides of identical sequence found in different proteins; in some cases the conformations of the pentapeptides are very different. Argos (ARGO87) surveyed pentapeptides of similar sequence in different proteins and found that the structures of the sequence-similar subsequences were frequently different.

The residues that join helices to helices, helices to sheets, and sheets to sheets are called turns and loops and have recently been classified by Richardson (RICH81), Thornton (THOR86), Sutcliffe et al. (SUTC87a) and others. Insertions and deletions are more readily tolerated in loops than elsewhere. Thornton et al. (THOR86) have summarized many observations indicating that related proteins usually differ most at the loops which join the more regular elements of secondary structure.

When the amino acid sequence of one protein has been changed to be more like the sequence of a second protein, the properties of the novel protein usually approach the properties of the second protein. Wells et al. (WELLS7a) reported that changing three residues in subtilisin from Bacillus amyloliquefaciens to be the

same as the corresponding residues in subtilisin from B. licheniformis produced a protease that had nearly the same activity as the subtilisin from the latter organism. There were 82 differences remaining in the sequences. The three residues changed were chosen because they were the only differences within 7 Angstroms (A) of the active site.

Many proteins bind non-covalently but very tightly and specifically to some other characteristic molecules. Schulz and Schirmer summarize many observations on the binding of proteins to other proteins (SCHU79, p98-105). For example, haemoglobin alpha chains bind very tightly to haemoglobin beta chains (delta G less than -11.0 Kcal/mole); antibodies bind tightly to antigens (K_ds range from 10^{-6} to 10^{-14} M, K_d is the dissociation constant equal to [A][B]/[A:B]); basic bovine pancreatic trypsin inhibitor (BPTI) binds tightly to trypsin (K_d = 6.0 x 10^{-14} M (TSCH87), delta G = -18.0 Kcal/mole); and avidin binds to biotin (K_d = 1.3 x 10^{-15} M (CREI84, p362)).

In each case the binding results from complementarity of the surfaces that come into contact: bumps fit into holes, unlike charges come together, dipoles align, and hydrophobic atoms contact other hydrophobic atoms. Although bulk water is excluded, individual water molecules are frequently found filling space in intermolecular interfaces; these waters usually form hydrogen bonds to one or more atoms of the protein or to other bound water. Thus proteins found in nature have not attained, nor do they require perfect complementarity to bind tightly and specifically to their substrates. Only in rare cases

is there essentially perfect complementarity; then the binding is extremely tight (as for example, avidin binding to biotin).

The relative importance of electrostatic vs. hydrophobic interactions is not fully understood (SCHU79, p105). Attraction between oppositely charged groups apparently contributes little to the free-energy of binding between proteins and other molecules. Like-charged groups can, however, increase specificity: repulsion of like-charged groups in the binding interface or even unpaired charges in the interface can greatly reduce or eliminate binding in instances where shape and hydrophobic interactions would otherwise induce it.

It has been observed, however, that proteins can bind to other molecules such that like-charged groups are juxtaposed; in such instances repulsion is reduced or eliminated by inclusion of oppositely charged ions in the binding interface. An example of this phenomenon is the inclusion of two positively charged calcium ions between each pair of subunits of turnip crinkle virus (HOGL83). The subunits each contain two negatively charged D (single-letter amino acid codes are given in Table 1) and E residues in close proximity.

The factors affecting protein binding are known, (CHOT75, CHOT76, SCHU79, p98-107, and CREI84, Ch8), but designing new complementary surfaces has proved difficult. Although some rules have been developed for substituting side groups (SUTC87b), the side groups of proteins are floppy and it is difficult to predict what conformation a new side group will take. Further, the

forces that bind proteins to other molecules are all relatively weak and it is difficult to predict the effects of these forces.

Recently, Quiocho and collaborators (QUIO87) elucidated the structures of several periplasmic binding proteins from Gram-negative bacteria. They found that the proteins, despite having low sequence homology and differences in structural detail, have certain important similarities. Each of the proteins they investigated is composed of two domains that are joined by three strands of protein. The binding site is located between the two domains and is isolated from bulk solvent. The structure of the binding site is dense and highly ordered, and binding constants are very high. The researchers suggest that binding of ligands causes a conformational change that alters the relative positions of the two domains.

The researchers found that each of the periplasmic binding proteins has numerous residues (seven or more), arrayed about the binding site. Surprisingly, ionic ligands are not bound by ionic side groups or opposite charge, but by main-chain components. Electrical charge seems to be neutralized by dipole interactions. Further, hydrophobic contacts play an important role in binding.

Based on their investigations of these binding proteins, Quiocho et al. suggest it is unlikely that, using current protein engineering methods, proteins can be constructed with binding properties superior to those of proteins that occur naturally.

Wilkinson et al. (WILK84) have found, however, that enzyme-substrate affinity may be increased by protein engineering. They reported that a mutant of tyrosyl tRNA synthetase of Bacillus stearothermophilus that has proline at residue 51 exhibits a 100-fold increase in affinity for ATP.

Substitution of one amino acid for another at a surface locus may profoundly alter binding properties of the protein other than substrate binding, without affecting the tertiary structure of the protein. For example, in sickle-cell haemoglobin the change of the surface residue E6 to V in the beta chains causes deoxyhaemoglobin-S to form fibers through self binding (DICK83, p125-145). Love and others have shown that the tertiary and quaternary structure of the haemoglobin are not changed (PADL85, WISH75, WISH76).

Tan and Kaiser (TANK77) and Tschesche et al. (TSCH87) showed that changing a single amino acid in BPTI greatly reduces its binding to trypsin, but that some of the new molecules retain the parental characteristics of binding to and inhibiting chymotrypsin, while others exhibit new binding to elastase. Caruthers and others (EISE85) have shown that changes of single amino acids on the surface of the lambda Cro repressor greatly reduce its affinity for the natural operator Or3, but greatly increase the binding of the mutant protein to a mutant operator. Thus changing the surface of a binding protein may alter its specificity without abolishing binding activity.

The recently developed techniques of "reverse genetics" have been used to produce single specific

mutations at precise base pair loci (OLIP86, OLIP87, and AUSU87). Mutations are generally detected by sequencing and in some cases by loss of wild-type function. These procedures allow researchers to analyze the function of each residue in a protein (MILL88) or of each base pair in a regulatory DNA sequence (CHEN88). In these analyses, the norm has been to strive for the classical goal of obtaining mutants carrying a single alteration (AUSU87).

Reverse genetics is frequently applied to coding regions to determine which residues are most important to the protein structure and function. In such studies, isolation of a single mutant at each residue of the protein gives an initial estimate of which residues play crucial roles.

Prior to the method of the present invention, two general approaches have been developed to create novel mutant proteins through reverse genetics. Both methods start with a clone of the gene of interest. In one approach, dubbed "protein surgery" (reviewed by Dill, (DILL87)), a specific substitution is introduced at a single protein residue by a synthetic method using the corresponding natural or synthetic cloned gene. Craik et al. (CRAI85), Roa et al. (RAOS87), and Bash et al. (BASH87) have used this approach to determine the effects on structure and function of specific substitutions in trypsin.

The other approach has been to generate a variety of mutants at many loci within the cloned gene, the "gene-directed random mutagenesis" method. The specific location and nature of the change are determined by DNA sequencing. It may be possible to

screen for mutations if loss of a wild-type function confers a cellular phenotype. Using immunoprecipitation, one can then differentiate among mutant proteins that: a) fold but fail to function, b) fail to fold but persist, and c) are degraded, perhaps due to failure to fold. This approach is exemplified by the work of Pakula et al. (PAKU86) on the effect of point mutations on the structure and function of the Cro protein from bacteriophage lambda. This approach is limited by the number of colonies that can be examined. An additional important limitation is that many desirable protein alterations require multiple amino acid substitutions and thus are not accessible through single base changes or even through all possible amino acid substitutions at any one residue.

The objective in both the surgical and gene-directed random mutagenesis approaches has been, however, to analyze the effects of a variety of single substitution mutations, so that rules governing such substitutions could be developed (ULME83). Progress has been greatly hampered by the extensive efforts involved in using either method and the practical limitations on the number of colonies that can be inspected (ROBE86).

The term "saturation mutagenesis" with reference to synthetic DNA is generally taken to mean generation of a population in which: a) every possible single-base change within a fragment of a gene of DNA regulatory region is represented, and b) most mutant genes contain only one mutation. Thus a set of all possible single mutations for a 6 base pair length of DNA comprises a population of 18 mutants. Oliphant et al. (OLIP86) and Oliphant and Struhl (OLIP87) have demonstrated ligation

and cloning of highly degenerate oligonucleotides and have applied saturation mutagenesis to the study of promoter sequence and function. They have suggested that similar methods could be used to study genetic expression of protein coding regions of genes, but they do not say how one should: a) choose protein residues to vary, or b) select or screen mutants with desirable properties.

Reidhaar-Olson and Sauer (REID88) have used synthetic degenerate oligo-nts to vary simultaneously two or three residues through all twenty amino acids in the dimer interface of cI repressor from bacteriophage lambda. They give no discussion of the limits on how many residues could be varied at once nor do they mention the problem of unequal abundance of DNA encoding different amino acids. They looked for proteins that either had wild-type dimerization or that did not dimerize. They did not seek proteins having novel binding properties and did not find any.

Several researchers have designed and synthesized proteins de novo. These designed proteins are small and most have been synthesized in vitro as polypeptides rather than genetically. Gutte and colleagues have made a polypeptide that binds DDT in 55% ethanol (MOSE83). Recently Moser et al. (MOSE87) reported genetic expression in E. coli both of the designed 24 residue DDT-binding protein and of fusions of the DDT-binding sequence to LacZ. They state that design of biologically active proteins is currently impossible.

Erickson et al. (ERIC86) have designed and synthesized a series of proteins that they have named betabellins, that are meant to have beta sheets. They

suggest use of polypeptide synthesis with mixed reagents to produce several hundred analogous betabellins. They suggest the mixture be passed over a column to recover the analogues with high affinity for a chosen target compound bound to the column. They envision successive rounds of mixed synthesis of variant proteins and purification by specific binding. They do not discuss how residues should be chosen for variation. Because proteins can not be amplified, the researchers must sequence the recovered protein to learn which substitutions improve binding. The researchers must limit the level of diversity so that each variety of protein will be present in sufficient quantity for the isolated fraction to be sequenced.

A number of methods have been developed to separate cells through their affinity to various substances. Bonnafous et al. (BONN85) review methods that have been applied to animal cells, and cite two common problems: a) non-specific interactions between cells and affinity supports, and b) irreversible binding of cells to affinity matrices. Possible reasons for irreversible binding include multiple points of attachment and very high affinity between cells and antibodies used as affinity materials. Chromatographic separation of animal cells is still difficult because of their fragility. Bacterial cells, bacterial spores, and some bacteriophage, however, are sturdier than animal cells and have been fractionated based on proteins displayed on their surfaces.

Ferenci and collaborators have published a series of papers on the chromatographic isolation of mutants of the maltose-transport protein LamB of E. coli (WAND79, FERE80a, FERE80b, FERE80c, FERE82a, FERE82b,

FERE81, CLUN34, FERE86a, FERE86b, FERE86c, FERE87a, FERE87b, HEIN87, and HEIN88). The papers report that spontaneous and induced mutants at the lamB genetic locus can be isolated by chromatography over a column supporting immobilized maltose, maltodextrins, or starch, i.e. carbohydrates that could be metabolized by the bacteria. The reports speculate that other applications are possible, but specifically mention only the elucidation of the residues responsible for the selectivity of the maltodextrin pore or similar pore proteins.

Ferenci's experiments measured the combination of the individual affinity of mutant LamB molecules and the level of expression. Several classes of mutants in lamB were isolated. One class had higher affinities for both maltose and starch, one class had lower affinity for starch but higher affinity for maltose, and another class had higher affinity for starch but lower affinity for maltose.

Mutants were generated either by hydroxylamine treatment of a plasmid carrying the entire gene, or by insertions of two extra codons at natural Hpa II sites. Levels of mutagenesis were picked to provide single point mutations or single insertions of two residues. No multiple mutations were sought or found.

LamB is a large trimeric integral membrane protein; such proteins are very difficult to crystallize or even to solubilize. Therefore it is difficult to use single-crystal protein X-ray crystallography or NMR to obtain detailed 3D structural information. Garavito et al. (GARA83) have obtained crystals of LamB that diffract X-rays, but the 3D

structure of the protein has not yet been determined. There are models (GEHR87, HEIN88) that include the secondary structure of LamB, i.e. they specify which residues are in beta-sheet conformation, which residues are in turns, and which residues are on the outside, in the periplasm, or in the membrane. These models do not specify how the beta sheets are arranged nor which turns are close to which other turns.

Ferenci and Lee (FERE86a) reported on the temperature sensitivity of carbohydrate binding in B. stearothermophilus. At higher temperatures, the organism breaks down the polysaccharide, the binding of which was the object of the study. Clune, Lee, and Ferenci (CLUN84) reported that presence of complete O-antigen affected the binding properties of LamB on the surface of E. coli. Both of these reports point up the difficulties of working with live bacteria that can metabolize chemicals and change their physiological behavior during the chromatographic experiment. Heine et al. (HEIN83) have used the chemotaxis of E. coli recently to isolate mutants in lamB that are unaffected in chemotaxis: this approach is limited to metabolites that affect chemotaxis.

Makela et al. (MAKE80) reviewed methods that involve chemically coupling antigens to bacteriophage to produce a sensitive, quantitative detection system for antibodies. The methods reviewed exploit the ability to amplify the signal produced by antibodies binding to the antigens coupled to the phage, through growth of the phage. The antigens were joined to the phage chemically and not encoded in the genes of the phage. Thus there was no sorting of genetic material. Furthermore, the objectives of the methods reviewed

involve titering the phage that fail to bind, as an assay of antibody. The methods of the present invention, in most cases, involve growth and amplification of genetic packages that bind with high affinity.

In 1985 Smith (SMIT85) reported inserting a heterologous gene into gene III of bacteriophage f1. The gene III protein is a minor coat protein necessary for infectivity. In some cases the inserted gene preserved the original reading frame, leading to expression of heterologous protein as an inserted domain in the gene III protein. Smith demonstrated that the resulting strain of f1 virion are adsorbed by antibody against the protein encoded by the heterologous DNA. The antibody was bound to a polystyrene petri dish. The phage were eluted at pH 2.2 and retained some infectivity. However, the single copy of f1 gene III was used for insertion of the heterologous gene so that all copies of gene III protein were affected: infectivity of the resultant phage was reduced 25-fold. Smith also demonstrated that batch elution from a plate can separate f1 virions that differ by only a few protein domains on their surfaces.

Smith presented his method as a way to isolate cloned genes using antibodies to the gene products. He made no mention of mutagenizing the inserted genetic material or of inducing novel binding properties in the inserted protein domain.

De la Cruz et al. (CRUZ88) have expressed a fragment of the repeat region of the circumsporozoite protein from Plasmodium falciparum on the surface of

M13 as an insert in the gene III protein. They showed that the recombinant phage were both antigenic and immunogenic in rabbits, and that such recombinant phage could be used for B epitope mapping. The researchers suggest that similar recombinant phage could be used for T epitope mapping and for vaccine development. They do not suggest mutagenesis of the inserted material.

Gene fragments coding for portions of hepatitis B virus antigens have been fused to fragments of lamB. If the point of fusion is in a region coding for exposed domains of LamB, the HBV antigens appear on the cell surface and are immunogenic (CHAR87). Charbit et al. (CHAR87) suggest use of these engineered strains for development of a live bacterial vaccine; they have not reported interest in mutagenesis of the fused heterologous gene fragments, nor in development of binding capabilities.

Recently Tjian and colleagues (NODA85, BRIG87, and JONE87) have shown that DNA of definite sequence bound to an affinity column can be used to purify proteins that bind the DNA sequence-specifically. The proteins are purified as much as 1000-fold in two chromatographic steps or 80-fold in a single step.

Patents and patent applications which may be of interest include US Patent No. 4,704,692, "Computer Based System and Method for Determining and Displaying Possible Chemical Structures for Converting Double- or Multiple-Chain Polypeptides to Single-Chain Polypeptides" (Ladner '692), issued to Robert Charles Ladner on 3 November 1987 and assigned to Genex Corporation. Ladner '692 describes a design method for

converting proteins composed of two or more chains into proteins of fewer polypeptide chains, but with essentially the same 3D structure. There is no mention of variegated DNA and no genetic selection.

Robert Charles Ladner also has six patent applications pending before the USPTO and assigned to Genex Corporation:

07/92,110
07/21,046
07/21,047
07/34,964
07/34,965
07/34,966

Sonia K. Guterman is named as a joint inventor on US patent No. 4,745,056 ("Streptomyces Secretion Vector") and on Ser. No. 21,465.

None of the Ladner or Guterman patents or applications is believed to disclose or suggest the present invention, but it is requested that each be considered by the Examiner.

No admission is made that any cited reference is prior art or pertinent prior art, and the dates given are those appearing on the reference and may not be identical to the actual publication date.

SUMMARY OF THE INVENTION

This invention relates to the construction, expression, and selection of mutated genes that specify novel proteins with desirable binding properties, as well as these proteins themselves. The substances

bound by these proteins, hereinafter referred to as "targets", may be, but need not be, proteins. Targets may include other biological or synthetic macromolecules as well as organic and inorganic molecules.

The novel binding proteins may be obtained: 1) by mutating a gene encoding a known binding protein within the subsequence encoding a known binding domain, or 2) by taking such a subsequence of the gene for a first protein and combining it with all or part of a gene for a second protein (which may or may not be itself a known binding protein), 3) by mutating a gene encoding a protein which, while not possessing a known binding activity, possesses a secondary or higher structure that lends itself to binding activity (clefts, grooves, etc.), or 4) by mutating a gene encoding a known binding protein but not in the subsequence known to cause the binding. The protein from which the novel binding protein is derived need not have any specific affinity for the target material.

In one embodiment, the invention relates to:

a) preparing a variegated population of replicable genetic packages, each package including a nucleic acid construct coding on expression for an outer-surface-displayed potential binding protein comprising (i) a structural signal directing the display of the protein on the outer surface of the package and (ii) a potential binding domain for binding said target, where a plurality of different potential binding domains are displayed by the individual packages;

The invention further relates to a method of preparing a mixed population of replicable genetic packages in which each package includes a gene expressing a potential binding protein in such a manner that the protein is presented on the outer surface of the package. This method comprises:

i) preparing a variegated population of DNA inserts of each of which comprises a first sequence which codes on expression for a potential binding domain and, a second sequence encoding signal directing that the encoded protein be displayed on the outer surface of a chosen replicable genetic package, and

ii) incorporating the resulting population of DNA constructs into the chosen replicable genetic packages to produce a population of replicable genetic packages.

In a preferred embodiment, the potential-binding-protein-encoding inserts are incorporated into a gene encoding an outer-surface protein of the replicable genetic package.

The invention encompasses the design and synthesis of variegated DNA encoding a family of potential binding proteins characterized by constant and variable regions, said proteins being designed with a view toward obtaining a protein that binds a predetermined target.

For the purposes of this invention, the term "potential binding protein" refers to a protein encoded by one species of DNA molecule in a population of

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For the purposes of this invention, the term "potential binding protein" refers to a protein encoded by one species of DNA molecule in a population of

proteins that in fact bind to the target ("successful binding domains"). After one or more rounds of such enrichment, one or more of the chosen genes are examined and sequenced. If desired, new loci of variation are chosen. The selected daughter genes of one generation then become the parent sequences for the next generation of variegated DNA, beginning the next "variegation cycle." Such cycles are continued until a protein with the desired target affinity is obtained.

The appended claims are hereby incorporated by reference into this specification as an enumeration of the preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic showing the relationships between various types of Binding Domains (BD).

Figure 2 is a flow chart showing the major steps used to create a novel protein with affinity for a predetermined target.

Figure 3 is a stereo view of a molecular model of the coat of the bacteriophage f1.

Figure 4 is a schematic of a PBD contacting a molecule of target material.

Figure 5 is a stereo view of a hypothetical interaction between BPTI and myoglobin.

Figure 6 is a schematic of the binding surface of a PBD at various stages in the process of selecting a successful binding domain for a hypothetical target.

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1.1 Bacterial Cells as Genetic Packages
1.1.1 Preferred Bacterial Cells for Use as GPs
1.1.2 Preferred Outer Surface Proteins for Displaying IPBDs on Bacterial Cells
1.1.3 Choice of Insertion site for IPBD in Bacterial Cell OSP
1.1.4 In Vivo Selection for Pseudo OSP Gene from Random DNA Inserts in Bacterial Cells

1.2 Displaying IPBD on bacterial spores
1.2.1 Preferred Bacterial Spores for Use as GPs
1.2.2 Preferred Outer-Surface Proteins for Displaying IPBD on Bacterial Spores
1.2.3.1 Choice of Insertion site for IPBD in OSP
1.2.4 In Vivo Selection for Pseudo OSP Gene from Random DNA Inserts in Bacterial Spores

1.3 Displaying IPBD on Outer Surface of Phages
1.3.1 Preferred Phages for Use as GPs
1.3.2 Preferred OSPs for Displaying IPBDs on Phages
1.3.3 Choice of Insertion site for IPBD in OSP
1.3.4 In Vivo Selection for Pseudo-OSP Gene from Random DNA Inserts in Phages

2. Choice of IPBD
2.1.1 Influence of target size on choice of IPBD
2.1.2 Influence of target charge on choice of IPBD
2.1.3 Other considerations in the choice of IPBD

3. Choice of OCV
4. Designing the osp-ipbd gene Insert
4.1 Genetic regulation of the osp-ipbd gene
4.2 DNA sequence design
4.3 Specific DNA sequence assignment

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13.1.2 The Secondary Set
13.1.3 Choice of Residues to Vary Initially
13.2 Choosing range of variation
13.3 Design of vg DNA Encoding PBD Family
14.1 Insertion of synthetic vgDNA into plasmids
14.2 Transformation of cells
14.3 Growth of the GP(vgPBD) population

15. Isolation of GP(SBD)s with binding-to-target phenotypes
15.1 Attaching the target material to a column
15.2 Reducing selection due to non-specific binding
15.3 Eluting the column
15.4 Recovery of packages
15.5 Amplifying the enriched packages
15.6 Determining whether further enrichment is needed
15.7 Characterizing population
15.8 Testing of binding affinity
15.9 Other Affinity Separation Means

16.0 The Next Variegation Cycle

17.0 OTHER CONSIDERATIONS
17.1 Joint selections
17.2 Selection for non-binding
17.3 Selection of PBDs for retention of structure
17.4 Created binding proteins not unique
17.5 Other modes of mutagenesis possible
Example 1 Derivation of Novel Binding Protein for Myoglobin Using BPTI as IPBD, M13 as GP, and the Gene VIII Protein as OSP.

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14.1 Insertion of synthetic vgDNA into plasmids
14.2 Transformation of cells
14.3 Growth of the GP(vgPBD) population

15. Isolation of GP(SBD)s with binding-to-target phenotypes
15.1 Attaching the target material to a column
15.2 Reducing selection due to non-specific binding
15.3 Eluting the column
15.4 Recovery of packages
15.5 Amplifying the enriched packages
15.6 Determining whether further enrichment is needed
15.7 Characterizing population
15.8 Testing of binding affinity
15.9 Other Affinity Separation Means

16.0 The Next Variegation Cycle

17.0 OTHER CONSIDERATIONS
17.1 Joint selections
17.2 Selection for non-binding
17.3 Selection of PBDs for retention of structure
17.4 Created binding proteins not unique
17.5 Other modes of mutagenesis possible
Example 1 Derivation of Novel Binding Protein for Myoglobin Using BPTI as IPBD, M13 as GP, and the Gene VIII Protein as OSP.

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bind a chosen target, it is referred to herein as a "binding domain" (BD). A preliminary operation is to engineer the appearance of a stable protein domain, denoted as an "initial potential binding domain" (IPBD), on the surface of a genetic package. The present invention is concerned with the expression of numerous, diverse, variant "potential binding domains" (PBD), all related to a "parental potential binding domain" (PPBD) such as the binding domain of a known binding protein, and with selection and amplification of the genes encoding the most successful mutant PBDs. An IPBD is chosen as PPBD to the first round of variegation. Selection-through-binding isolates one or more "successful binding domains" (SBD). An SBD from one round of variegation and selection-through-binding is chosen to be the PPBD for the next round. The invention is not, however, limited to proteins with a single BD since the method may be applied to any or all of the BDs of the protein, sequentially or simultaneously. The relationships of the various BDs are illustrated in Figure 1.

Conventionally, DNA sequences are written from 5' to 3', left-to-right showing only the sequence that will appear as mRNA (with each T of DNA changed to U in mRNA).

protein: M - L - F -

anti-sense DNA: 5' ATG CTT TTC ... 3'
sense DNA: 3' TAC GAA AAG ... 5'

mRNA: 5' AUG CUU UUC ... 3'

The complementary strand is the one used as template for mRNA synthesis and so is called the "sense strand"; we will use this convention throughout. Although this

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the analyte can be freed from the affinity material once the impurities are washed away.

Affinity column chromatography involves chemically attaching the affinity material to an inert solid support matrix that is held in a column so that solutions can be passed over the matrix in a controlled way. Mixtures that might contain the analyte are passed over the matrix to which any analyte component in the mixture adheres. Separation is achieved by passing a gradient of some type over the matrix and collecting fractions. It is also possible to recover purified material from the matrix by other means after impurities have been washed away.

An alternative to column affinity chromatography is batch elution from an affinity matrix material held in some container. Affinity material is chemically bound to the matrix. A mixture that might contain the analyte is added and the matrix is rinsed with buffer. The material is rinsed with a series of buffers containing increasing concentrations of solutes chosen to wash impurities away. The analyte is recovered in purified form either in one of the buffer fractions or bound to the matrix.

Another alternative to column affinity chromatography is batch elution from a plate. The affinity material can be chemically bound to a flat surface, such as the bottom of a polystyrene petri dish. A mixture that might contain the analyte is added to the plate and the plate is rinsed with a buffer. Subsequently, the plate is washed with a series of buffers containing increasing concentrations of solutes chosen to separate components having lower affinity for

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The present invention makes use of affinity separation of bacterial cells, or bacterial viruses (or other genetic packages) to enrich a population for those cells or viruses carrying genes that code for proteins with desirable binding properties.

In the present invention, the words "grow", "growth", "culture", and "amplification" mean increase in number, not increase in size of individual cells or phage. In the present invention, the words "select" and "selection" are used in the genetic sense: i.e. a biological process whereby a phenotypic characteristic is used to enrich a population for those organisms displaying the desired phenotype. Choices or elections to be made by humans are indicated by "choose", "pick", "take", etc., but not "select".

The process of the present invention comprises three major parts:

I. design and production of a replicable genetic package (GP) that displays an IPBD on the surface of the GP; the combination is denoted GP(IPBD).

II. design and implementation of an affinity separation process that separates GP(IPBD)s that bind to a known affinity molecule from wild-type GPs or GP(IPBD$^-$)s, neither of which binds the known affinity molecule, and

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II. design and implementation of an affinity separation process that separates GP(IPBD)s that bind to a known affinity molecule from wild-type GPs or GP(IPBD$^-$)s, neither of which binds the known affinity molecule, and

3) designing an amino acid sequence that: a) includes the IPBD as a subsequence and b) will cause the IPBD to appear on the GP surface (Secs. 1.1.2, 1.2.2, 1.3.2, and 4),

4) engineering a gene, denoted opmimpd, that: a) codes for the designed amino acid sequence, b) provides the necessary genetic regulation, and c) introduces convenient sites for genetic manipulation (Secs. 4.1, 4.2, 4.3, 5.1, and 5.2),

5) cloning the osp-ipbd gene into the GP (Sec. 6.1), and

6) harvesting the transformed GPs (Sec. 7) and testing them for presence of IPBD on the GP surface (Sec. 8); this test is performed with an affinity molecule having high affinity for IPBD, denoted AfM(IPBD).

In another preferred embodiment, Part I of the process involves:

1) choosing a GP such as a bacterial cell (Sec. 1.1.1), bacterial spore (1.2.1), or phage (1.3.1) having a suitable outer surface protein (Secs. 1.1.2, 1.2.2 and 1.3.2),

2) choosing a stable IPBD (Sec. 2),

3) designing a DNA sequence that: a) encodes the IPBD as a subsequence and b) contains suitable restriction sites so that random DNA may be operably linked to the ipbd gene fragment; and c)

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domain. References to PBD or pbd in Part I are to indicate a preparatory intent.

In Part II we optimize separation of GP(IPBD) from wild-type GP, denoted wtGP, based on the affinity of IPBD for AfM(IPBD). To establish the sensitivity of the affinity separation process, we separate small amounts of GP(IPBD) from much larger amounts of wtGP. In a preferred embodiment, Part II of the process of the present invention involves:

1) preparing affinity columns bearing AfM(IPBD) at various densities of AfM(IPBD)/(volume of matrix), (Sec. 10.1),

2) preparing GP(IPBD)s with various amounts of IPBD per GP,

3) picking a gradient regime for eluting the columns (Sec. 10.1),

4) determining which combination of: a) IPBD/GP, b) density of AfM(IPBD)/(volume of support), c) initial ionic strength, d) elution rate, and e) (amount of GP)/(volume of support) loaded, gives the best separation of GP(IPBD) from wtGP (Sec. 10.1),

5) determining the smallest amount of GP(IPBD) that can be isolated from a much larger amount of wtGP using the optimal condition, (Sec. 10.2), and

6) determining the efficiency of the affinity separation procedure (Sec. 10.3).

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6) determining the efficiency of the affinity separation procedure (Sec. 10.3).

3) picking a set of several residues in the PPBD to vary; the principal indicators of which residues to vary include: a) the 3D structure of the PPBD, b) sequences of homologous proteins, and c) computer or theoretical modeling that indicates which residues can tolerate different amino acids without disrupting the underlying structure (Sec. 13.1),

4) picking a subset of the residues picked in Part III.3, to be varied simultaneously (Sec. 13.1); the principal considerations are the number of different variants and which variants are within the detection capabilities of the affinity separation determined in Part II, and setting the range of variation (Sec. 13.2);

5) implementing the variegation by:

a) synthesizing the part of the osp-pbd gene that encodes the residues to be varied using a specific mixture of nucleotide substrates for some or all of the bases encoding residues slated for variation, thereby creating a population of DNA molecules, denoted vgDNA (Sec. 13.3),

b) ligating this vgDNA, by standard methods, into the operative cloning vector (OCV) (e.g. a plasmid or bacteriophage) (Sec. 14.1),

c) using the ligated DNA to transform cells, thereby producing a population of transformed cells (Sec. 14.2),

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5) implementing the variegation by:

a) synthesizing the part of the osm-pbd gene that encodes the residues to be varied using a specific mixture of nucleotide substrates for some or all of the bases encoding residues slated for variation, thereby creating a population of DNA molecules, denoted vgDNA (Sec. 13.3),

b) ligating this vgDNA, by standard methods, into the operative cloning vector (OCV) (e.g. a plasmid or bacteriophage) (Sec. 14.1),

c) using the ligated DNA to transform cells, thereby producing a population of transformed cells (Sec. 14.2),

Abbreviation	Meaning
GP	Genetic Package, e.g. a bacteriophage
wtGP	Wild-type GP
X	Any protein
X	The gene for protein X
IPBD	Initial Potential Binding Domain, e.g. BPTI
PBD	Potential Binding Domain, e.g. a derivative of BPTI
SBD	Successful Binding Domain, e.g. a derivative of BPTI selected for binding to a target
PPBD	Parental Potential Binding Domain, i.e. an IPBD or an SBD from a previous selection
OSP	Outer Surface Protein, e.g. coat protein of a phage or LamB from E. coli
OSP-PBD	Fusion of an OSP and a PBD, order of fusion not specified
OSTS	Outer Surface Transport Signal

GP(x)	A genetic package containing the x gene
GP(X)	A genetic package that displays X on its outer surface
GP(osp-pbd)	GP containing an osp-pbd gene
GP(OSP-PBD)	A genetic package that displays PBD on its outside as a fusion to OSP
GP(pbd)	GP containing a pbd gene, osp implicit
GP(PBD)	A genetic package displaying PBD on its outside, OSP unspecified
(Q)	An affinity matrix supporting "Q", e.g. (T4 lysozyme) is T4 lysozyme attached to an affinity matrix
AfM(W)	A molecule having affinity for "W", e.g. trypsin is an AfM(BPTI)
AfM(W)*	AfM(W) carrying a label, e.g. ^{125}I
XINDUCE	A chemical that can induce

	expression of a gene, e.g. IPTG for the lacUV5 promoter
OCV	Operative Cloning Vector
K_T	$K_T = [T][SBD]/[T:SBD]$ (T is a target)
K_N	$K_N = [N][SBD]/[N:SBD]$ (N is a non-target)
DoAMoM	Density of AfM(W) on affinity matrix
mfaa	Most-Favored amino acid
lfaa	Least-Favored amino acid
Abun(x)	Abundance of DNA molecules encoding amino acid x
OMP	Outer membrane protein
nt	nuclectide
K_d	A bimolecular dissociation constant, $K_d = [A][B]/[A:B]$
SP-I	Signal-sequence Peptidase I
Y_{DQ}	Yield of ssDNA up to Q bases long
M_{DNA}	Maximum length of ssDNA that

	can be synthesized in acceptable yield
Y_{pl}	Yield of plasmid DNA per volume of culture
L_{eff}	DNA ligation efficiency
M_{ntv}	Maximum number of transformants produced from Y_{D100} DNA of Insert
C_{eff}	Efficiency of chromatographic enrichment, enrichment per pass
C_{sensi}	Sensitivity of chromatographic separation, can find 1 in N,
N_{chrom}	Maximum number of enrichment cycles per variegation cycle
S_{err}	Error level in synthesizing vgDNA

Sec. 0.3: Standard sequencing method:

The present invention is not limited to a single method of determining the sequence of nucleotides (nts) in DNA subsequences. In the preferred embodiment, plasmids are isolated and denatured in the presence of a sequencing primer, about 20 nts long, that anneals to a region adjacent, on the 5' side, to the region of interest. This plasmid is then used as the template in

the four sequencing reactions with one dideoxy substrate in each. Sequencing reactions, agarose gel electrophoresis, and polyacrylamide gel electrophoresis (PAGE) are performed by standard procedures (AUSU87).

The present invention is not limited to a single method of determining protein sequences, and reference in the appended claims to determining the amino acid sequence of a domain is intended to include any practical method or combination of methods, whether direct or indirect. The preferred method, in most cases, is to determine the sequence of the DNA that encodes the protein and then to infer the amino acid sequence. In some cases, standard methods of protein-sequence determination may be needed to detect post-translational processing.

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The major steps in the process of making and isolating a novel binding protein with affinity for a chosen target material are illustrated in Figure 2.

Sec. 1: Specification of Genetic Package and Means for Displaying a Heterologous Binding Domain On Its Outer Surface:

Sec. 1.0: General Requirements for Genetic Packages

It is emphasized that the GP on which selection-through-binding will be practiced must be capable, after the selection, either of growth in some suitable environment or of *in vitro* amplification and recovery of the encapsulated genetic message. During at least part of the growth, the increase in number must be

approximately exponential with respect to time. The component of a population that exhibits the desired binding properties may be quite small, for example, one in 10^6 or less. Once this component of the population is separated from the non-binding components, it must be possible to amplify it. Culturing viable cells is the most powerful amplification of genetic material known and is preferred. Genetic messages can also be amplified in vitro, but this is not preferred.

A GP may typically be a vegetative bacterial cell, a bacterial spore or a bacterial DNA virus. A strain of any living cell or virus is potentially useful if the strain can be:

1) maintained in culture,

2) affinity separated and retain its viability,

3) genetically altered with reasonable facility, and

4) manipulated to display the potential binding protein domain where it can interact with the target material during affinity separation.

We believe that it is possible to cause a genetic package to display the IPBD or PBD on its outer surface without adversely affecting the viability of the GP or the binding characteristics of the IPBD or PBD.

It is generally believed that the part of the polypeptide chain composing one domain folds almost independently of the parts composing other domains. There are natural proteins composed of two or more

domains for which there is strong evidence that essentially the same domain occurs more than once, for example ovomucoids and ovoinhibitors (SCOT87) and kallikrein (CHUN86). Furthermore, essentially the same domain can occur in several different proteins (SUDH85, GIL885, and SCOT87).

Rossman (ROSS81) and others have pointed out that the 3D structure of individual domains can be preserved during protein evolution, even after the amino acid sequences have diverged so much that no significant homology can be detected. Hollecker and Creighton (HOLL83) studied the folding pathways of two black mamba venom proteins (called I and K) that are homologous to BPTI. Although the sequences of I and K are clearly related to BPTI by the identity of 19 and 23 residues respectively, including all six cysteine residues, there are 38 and 34 differences. Not only are the 3D structures of the proteins very similar, but the pathway of folding has also been conserved.

When gene fragments coding for two domains from different proteins have been joined by genetic engineering and expressed, the domains from the original proteins sometimes fold independently while tethered to each other (TOTH86, SMIT85, MANO86). If the insertion is the gene for the entire protein, that protein may be converted into a domain of the larger protein. Fusions of genes that determine the domains, however, must be done at or near domain junctions, or domain function may be impaired (CRAW87, TOTH86). In some cases, the inserted domain will fold, but the recipient protein will not: Beckwith's fusions of malF and phoA genes (BECK81, MANO86) gave rise to functional PhoA domains attached to a fragment of MalF that

anchored the chimeric protein in the lipid bilayer. The MalF protein was incomplete and could not function.

There are two basic methods of arranging that the ipbd gene is expressed in such a manner that the IPBD is displayed on the outer surface of the GP.

First, DNA encoding the IPBD sequence may be operably linked to DNA encoding all or part of an outer surface protein (OSP) native to the GP. If one or more fusions of fragments of x genes to fragments of a natural osp gene are known to cause X protein domains to appear on the GP surface, then we pick the DNA sequence in which an ipbd gene fragment replaces the x gene fragment in one of the successful osp-x fusions as a preferred gene to be tested for the display-of-IPBD phenotype. (The gene may be constructed in any manner.) If no fusion data are available, then we fuse an ipbd fragment to various fragments, such as fragments that end at known or predicted domain boundaries, of the osp gene and obtain GPs that display the osp-ipbd fusion on the GP outer surface by screening or selection for the display-of-IPBD phenotype. The fusion of ipbd and osp fragments may also include fragments of random or pseudorandom DNA to produce a population, members of which may display IPBD on the GP surface. The members displaying IPBD are isolated by screening or selection for the display-of-binding phenotype.

While most bacterial proteins remain in the cytoplasm, others are transported to the periplasmic space (which lies between the plasma membrane and the cell wall of gram-negative bacteria), or are conveyed and anchored to the outer surface of the cell. Still

others are exported (secreted) into the medium surrounding the cell. These characteristics of a protein that are recognized by a cell and that cause it to be transported out of the cytoplasm and displayed on the cell surface will be termed "outer-surface transport signals".

It is believed that the conditions for an outer surface transport signal are not particularly stringent, i.e., a random polypeptide of appropriate length (preferably 30-100 amino acids) has a reasonable chance of providing such a signal. Thus, by constructing a chimeric gene comprising a segment encoding the IPBD linked to a segment of random or pseudorandom DNA (the potential OSTS), and placing this gene under control of a suitable promoter, there is a possibility that the chimeric protein so encoded will function as an OSP-IPBD.

This possibility is greatly enhanced by constructing numerous such genes, each having a different potential OSTS, cloning them into a suitable host, and selecting for transformants bearing the IPBD (or other marker) on their outer surface.

The replicable genetic entity (phage or plasmid) that carries the osp-pbd genes (derived from the osp-ipbd gene) through the selection-through-binding process, see Sec. 14, is referred to hereinafter as the operative cloning vector (OCV). When the OCV is a phage, it may also serve as the genetic package. The choice of a GP is dependent in part on the availability of a suitable OCV and suitable OSP.

Preferably, the GP is readily stored, for example, by freezing. If the GP is a cell, it should have a short doubling time, such as 20-40 minutes. If the GP is a virus, it should be prolific, e.g., a burst size of at least 100/infected cell. GPs which are finicky or expensive to culture are disfavored. The GP should be easy to harvest, preferably by centrifugation. The GP is preferably stable for a temperature range of -70 to 42°C (stable at 4°C for several days or weeks); resistant to shear forces found in HPLC; insensitive to UV; tolerant of desiccation; and resistant to a pH of 2.0 to 10.0, surface active agents such as SDS or Triton, chaotropes such as 4M urea or 2M guanidinium HCl, common ions such as K^+, Na^+, and SO_4^{--}, common organic solvents such as ether and acetone, and degradative enzymes. Finally, there must be a suitable OCV (see Sec. 3).

Although knowledge of specific OSPs may not be required for vegetative bacterial cells and endospores, the user of the present invention, preferably, will know: Is the sequence of any osp known? (preferably yes, at least one required for phage). How does the OSP arrive at the surface of GP? (knowledge of route necessary, different routes have different uses, no route preferred per se). Is the OSP post-translationally processed? (no processing most preferred, predictable processing preferred over unpredictable processing). What rules are known governing this processing, if there is any processing? (no processing most preferred, predictable processing acceptable). What function does the OSP serve in the outer surface? (preferably not essential). Is the 3D structure of an OSP known? (highly preferred). Are fusions between fragments of osp and a fragment of x

known? Does expression of these fusions lead to X appearing on the surface of the GP? (fusion data is as preferred as knowledge of a 3D structure). Is a "2D" structure of an OSP available? (in this context, a "2D" structure indicates which residues are exposed on the cell surface). (2D structure less preferred than 3D structure). Where are the domain boundaries in the OSP? (not as preferred as a 2D structure, but acceptable). Could IPBD go through the same process as OSP and fold correctly? (IPBD might need prosthetic groups) (preferably IPBD will fold after same process). Is the sequence of an osp promoter known? (preferably yes). Is osp gene controlled by regulatable promoter available? (preferably yes). What activates this promoter? (preferably a diffusible chemical, such as IPTG). How many different OSPs do we know? (the more the better). How many copies of each OSP are present on each package? (more is better).

The user will want knowledge of the physical attributes of the GP: How large is the GP? (knowledge useful in deciding how to isolate GPs) (preferably easy to separate from soluble proteins such as IgGs). What is the charge on the GP? (neutral preferred). What is the sedimentation rate of the GP? (knowledge preferred, no particular value preferred).

The preferred GP, OCV and OSP are those for which the fewest serious obstacles can be seen, rather than the one that scores highest on any one criterion.

Next, we consider general answers to the questions posed in this step for the cases of: a) vegetatively growing bacterial cells (Sec. 1.1), b) bacterial spores

(Sec. 1.2), and c) (Sec. 1.3). Preferred OSPs for several GPs are given in Table 2.

Sec. 1.1: Bacterial Cells as Genetic Packages:

One may choose any well-characterized bacterial strain which may be grown in culture. The important questions in this case are: a) do we know enough about mechanisms that localize proteins on the outside of the cell, b) will the IPBD fold in the environment of the outer membrane, and c) will cells change expression of osp-pbd, derived from osp-ipbd, during affinity separation? Some IPBDs may need large or insolubie prosthetic groups, such as haem or an Fe_4S_4 cluster, that are available within the cell, but not in the medium. The formation of Fe_4S_4 clusters found in some ferredoxins is catalyzed by enzymes found in the cell (BONG35). IPBDs that require such prosthetic groups may fail to fold or function if displayed on bacterial cells.

Sec. 1.1.1: Preferred Bacterial Cells as GP :

The species chosen should have a well-characterized genetic system and strains defective in genetic recombination should be available. The chosen strain may need to be manipulated to prevent changes of its physiological state that would alter the number or type of proteins or other molecules on the cell surface during the affinity separation procedure. In view of the extensive knowledge of E. coli, a strain of E. coli, defective in recombination, is the strongest candidate as a bacterial GP. Other preferred candidates are Salmonella typhimurium, Bacillus subtilis, and Pseudomonas aeruginosa.

Induction of synthesis of engineered genes in vegetative bacterial cells has been exercised through the use of regulated promoters such as lacUV5, trpP, or tac (MANI82). The factors that regulate the quantity of protein synthesized include: a) promoter strength (cf. HOOP87), b) rate of initiation of translation (cf. GOLD87), c) codon usage, d) secondary structure of mRNA, including attenuators (cf. LAND87) and terminators (cf. YAGE87), e) interaction of proteins with mRNA (cf. MCPH86, MILL87b, WINT87), f) degradation rates of mRNA (cf. SUBB88), g) proteolysis (cf. GOTT87). These factors are sufficiently well understood that a wide variety of heterologous proteins can now be produced in E. coli or B. subtilis in at least moderate quantities (SKER88, BETT86).

Sec. 1.1.2: Preferred Outer Surface Proteins for Displaying IPSPs on Bacterial Cells:

Gram-negative bacteria have outer-membrane proteins (OMP), that form a subset of OSPs. Many OMPs span the membrane one or more times. The signals that cause OMPs to localize in the outer membrane are encoded in the amino acid sequence of the mature protein. Fusions of fragments of ompA genes with fragments of an x gene have led to X appearing on the outer membrane (BENS84, CLEM81). The rules that govern the localization of OMP-X fusion proteins are not yet fully elucidated. Many OMPs are polymeric and non-essential; a non-essential OMP is preferred. A non-essential OMP for which there is knowledge of which residues are on the cell surface is more preferred. A non-essential OMP for which there is data showing that X is displayed as part of an OMP-X fusion is most

preferred. If no fusion data are available, then we fuse an ipbd fragment to various fragments of the osp gene and obtain GPs that display the osp-ipbd fusion on the cell outer surface by screening or selection for the display-of-IPBD phenotype.

Oliver has reviewed mechanisms of protein secretion in bacteria (OLIV85 and OLIV87). Nikaido and Vaara (NIKA87) have reviewed mechanisms by which proteins become localized to the outer membrane of Gram-negative bacteria. For example, the LamB protein of E. coli is synthesized with a typical signal-sequence which is subsequently removed. Benson et al. (BENS84) showed that LamB-LacZ fusion proteins would be deposited in the outer membrane of E. coli when residues 1-49 of the mature LamB protein are included in the fusion, but that residues 1-43 are insufficient. The rules that govern localization of proteins in the outer membrane of Gram-negative bacteria remain vague. Kaiser et al. (KAIS87) showed that the export signal in Saccharomyces cerevisiae is very broad, because when they fused random human DNA sequences to DNA coding for mature invertase, about one fifth of the sequences resulted in the appearance of invertase free in the medium.

The outer membrane protein LamB of E. coli is a porin for maltose and maltodextrin transport, and serves as the receptor for adsorption of bacteriophages lambda and K10. This protein has been purified to homogeneity (ENDE78) and shown to function as a trimer (PALV79). Mutations to phage resistance have been used to define the parts of the LamB protein that adsorb each phage (ROAM80, CLEM81, CLEM81, GEHR87). Phage-resistance mutations are dominant (MARC83), suggesting

that there is no preferential assembly of wild-type or mutant subunits.

In lamB$^+$ cells, addition of maltose or maltodextrin inhibits a form of motility called cell swarming, and lamB mutants defective in this process have been characterized (HEIN88). These mutations have been sequenced and compared to the wild-type sequence (CLEM81) and the concomitant protein domains have been analyzed (CLEM83). Topological models have been developed that describe the function of phage receptor and maltodextrin transport. The models describe these domains and their locations with respect to the surfaces of the outer membrane (CHAR84, HEIN88).

LamB is transported to the outer membrane if a functional N-terminal sequence is present; further, the first 49 amino acids of the mature sequence are required for successful transport (BENS84). Homology between parts of LamB protein and other outer membrane proteins OmpC, OmpF and PhoE has been detected (NIKA84), including homology between LamB amino acids 39-49 and sequences of the other proteins. These subsequences may label the proteins for transport to the outer membrane. Further, monoclonal antibodies derived from mice immunized with purified LamB, have been used to characterize four distinct topological and functional regions, two of which are concerned with maltose transport (GABA82).

General knowledge on processing of signal sequences in E. coli is relevant to the present invention both for use of E. coli per se and for use in conjunction with filamentous phage (vide infra). Genetic experiments on processing of signal sequences

indicate that if the S21-F22-A23 sequence is preserved, signal peptidase (SP-I) will cleave after A23 (OLIV87). Many examples have been cited in which the DNA coding for the leader or signal sequence from one protein has been attached to the DNA sequence coding for another protein, protein X (BECK83, INOU86 CH10, LEEC86, MARK86, and BOQU87). Expression of such a chimeric gene often causes protein X to appear free in the periplasm. That is, the leader causes the new protein to be secreted through the lipid bilayer: once in the periplasm, it is cleaved off by SP-I.

Beckwith (BECK83 and MANO86) has shown that when the phoA gene is inserted in frame into the coding sequence for an integral membrane protein, for example MalF, that the PhoA domain is localized according to where in the integral membrane protein the phoA gene was inserted. That is, if phoA is inserted after an amino acid which normally is found in the cytoplasm, then PhoA appears in the cytoplasm. If phoA is inserted after an amino acid normally found in the periplasm, however, then the PhoA domain is localized on the periplasmic side of the membrane, and anchored in it.

Beckwith and colleagues (BECK88) have extended these observations to the lacZ gene that can be inserted into genes for integral membrane proteins such that the LacZ domain appears in either the cytoplasm or the periplasm according to where the lacZ gene was inserted.

Sec. 1.1.3 Choice of Insertion site for IPBD in Bacterial Cell OSP:

OSP-IPBD fusion proteins need not fill a structural role in the outer membranes of Gram-negative bacteria because parts of the outer membranes are not highly ordered. For large OSPs there is likely to be one or more sites at which *osp* can be truncated and fused to *ipbd* such that cells expressing the fusion will display IPBDs on the cell surface. If fusions between fragments of *ozp* and *x* have been shown to display X on the cell surface, we can design an *osp-ipbd* gene by substituting *ipbd* for *x* in the DNA sequence. Otherwise, successful OMP-IPBD fusion is preferably sought by fusing fragments of the best *omp* to an *ipbd*, expressing the fused gene, and testing the resultant GPs for display-of-IPBD phenotype. We use the available data about OMP to pick the point or points of fusion between *omp* and *ipbd* to maximize the likelihood that IPBD will be displayed. Alternatively, we truncate *ozp* at several sites or in a manner that produces *osp* fragments of variable length and use the *osp* fragments to *ipbd*; cells expressing the fusion are screened or selected which display IPBDs on the cell surface. An additional alternative is to include short segments of random DNA in the fusion of *ozp* fragments to *ipbd* and then screen or select the resulting variegated population for members exhibiting the display-of-IPBD phenotype.

The promoter for the *osp-ipbd* gene, preferably, is subject to regulation by a small chemical inducer, such as isopropyl thiogalactoside (IPTG) (*lac* promoter). It need not come from a natural *osp* gene; any regulatable bacterial promoter can be used.

Once a genetic packaging system employing

vegetative bacterial cells has been designed, it is time to choose an IPBD (Sec. 2).

Sec. 1.1.4: In Vivo Selection for Pseudo-osp Gene From Random DNA Inserts in Bacterial Cells:

As an alternative to choosing a natural OSP and an insertion site in the OSP, we can construct a gene comprising: a) a regulatable promoter (e.g. lacUV5), b) a Shine-Dalgarno sequence, c) a periplasmic transport signal sequence, d) a fusion of the *inbd* gene with a segment of random DNA (as in Kaiser *et al.* (KAIS87)), e) a stop codon, and f) a transcriptional terminator. As previously stated, the purpose of the random DNA is to encode an OSTS, like that embodied in known OSPs. The fusion of *inbd* and the random DNA could be in either order, but *inbd* upstream is slightly preferred. Isolates from the population generated in this way can be screened for display of the IPBD. Preferably, a version of selection-through-binding is used to select GPs that display IPBD on the GP surface. Alternatively, clonal isolates of GPs may be screened for the display-of-IPBD phenotype.

The preference for *inbd* upstream of the random DNA arises from consideration of the manner in which the successful GP(IPBD) will be used. In Part III, we will introduce numerous mutations into the *pbd* region of the *osp-pbd* gene, some of which might include gratuitous stop codons. If *pbd* precedes the random DNA, then gratuitous stop codons in *pbd* lead to no OSP-PBD protein appearing on the cell surface. If *pbd* follows the random DNA, then gratuitous stop codons in *pbd* might lead to incomplete OSP-PBD proteins appearing on the cell surface. Incomplete proteins often are non-

specifically sticky so that GPs displaying incomplete PBDs are easily removed from the population.

The random DNA can be generated from any DNA having high sequence diversity by partially digesting with an enzyme that cuts very often. Sau3A I, for example, generates cohesive-ended DNA that can be cloned into a BamH I or Bgl II site. Alternatively, one could shear DNA having high sequence diversity, blunt the sheared DNA with the large fragment of E. coli DNA polymerase I (hereinafter referred to as Klenow fragment), and clone the sheared and blunted DNA into blunt sites of the vector (MANI82, p295, AUSU87: 5.1.1).

Sec. 1.2: Displaying IPBD on bacterial spores:

Bacterial spores have desirable properties as GP candidates. Bacillus spores neither actively metabolize nor alter the proteins on their surface. However, spores are much more resistant than vegetative bacterial cells or phage to chemical and physical agents. Spores have the disadvantage that the molecular mechanisms that trigger sporulation are less well worked out than is the formation of M13 or the export of protein to the outer membrane of E. coli.

Sec. 1.2.1.: Preferred Bacterial Spores for Use as GPs:

Bacteria of the genus Bacillus form endospores that are extremely resistant to damage by heat, radiation, desiccation, and toxic chemicals (reviewed by Losick et al. (LOSI86)). These spores have complex structure and morphogenesis that is species-specific and only partially elucidated. The following observations are relevant to the use of Bacillus spores

as genetic packages for the purposes of the present invention.

Plasmid DNA is commonly included in spores. Plasmid encoded proteins have been observed on the surface of Bacillus spores (DEBR86). Sporulation involves complex temporal regulation that is now moderately well understood (LOSI86). Special sigma factors, such as sigma^E, are produced during sporulation. RNA polymerase bound to a sporulation sigma factor recognizes promoters that are not recognized by RNA polymerase bound to a vegetative sigma factor. The sequences of several sporulation promoters are known; coding sequences operatively linked to such promoters are expressed only during sporulation. Ray et al. (RAYC87) have shown that the G4 promoter of B. subtilis is directly controlled by RNA polymerase bound to sigma^E.

Donovan et al. have identified several polypeptide components of B. subtilis spore coat (DONO87); the sequences of two complete coat proteins and amino-terminal fragments of two others have been determined. Some components of the spore are synthesized in the forespore, e.g. small acid-soluble spore proteins (ERRI88), while other components are synthesized in the mother cell and appear in the spore (e.g. the coat proteins). This spatial organization of synthesis is controlled at the transcriptional level.

Spores self-assemble, but the signals that cause various proteins to localize in different parts of the spore are not well understood; presumably, the signals controlling deposition of the coat proteins from the cytoplasm of the mother cell onto the spore coat are

embedded in the polypeptide sequence. Some, but not all, of the coat proteins are synthesized as precursors and are then processed by specific proteases before deposition in the spore coat (DONO87). Viable spores that differ only slightly from wild-type are produced in B. subtilis even if any one of four coat proteins is missing (DONO87). Disulfide bonds form within the spore (thiol reducing agents are needed to solubilize several of the proteins of the coat). The 12kd coat protein, CotD, contains 5 cysteines. CotD also contains an unusually high number of histidines (16) and prolines (7). The 11kd coat protein, CotC, contains only one cysteine and one methionine. CotC has a very unusual amino-acid sequence with 19 lysines (K) appearing as 9 K-K dipeptides and one isolated K. There are also 20 tyrosines (Y) of which 10 appear as 5 Y-Y dipeptides. Peptides rich in Y and K are known to become crosslinked in oxidizing environments (DEVO78, WAIT83, WAIT85, WAIT86). CotC contains 16 D and E amino acids that nearly equals the 19 Ks. There are no A, F, R, I, L, N, P, Q, S, or W amino acids in CotC. Neither CotC nor CotD is post-translationally cleaved. The proteins CotA and CotB are post-translationally cleaved.

Endospores from the genus Bacillus are more stable than are exospores from Streptomyces. Bacillus subtilis forms spores in 4 to 6 hours, but Streptomyces species may require days or weeks to sporulate. In addition, genetic knowledge and manipulation is much more developed for B. subtilis than for other spore-forming bacteria. Thus Bacillus spores are preferred over Streptomyces spores. Bacteria of the genus Clostridium also form very durable endospores, but clostridia, being strict anaerobes, are not convenient

to culture. The choice of a species of Bacillus is governed by knowledge and availability of cloning systems and by how easily sporulation can be controlled. A particular strain is chosen by the criteria listed in Sec. 1.0. Spores are exposed to an oxidative environment after release from the mother cell, so that disulfides, if any, within the IPBD might form. Many vegetative biochemical pathways are shut down when sporulation begins so that prosthetic groups might not be available.

Sec. 1.2.2 Preferred outer-surface proteins for Displaying IPBD on Bacterial Spores:

If a spore is chosen as GP, the promoter is the most important part of the ocp gene, because the promoter of a spore coat protein is most active: a) when spore coat protein is being synthesized and deposited onto the spore and b) in the specific place that spore coat proteins are being made. In B. subtilis, some of the spore coat proteins are post-translationally processed by specific proteases. It is valuable to know the sequences of precursors and mature coat proteins so that we can avoid incorporating the recognition sequence of the specific protease into our construction of an OSP-IPBD fusion. The sequence of a mature spore coat protein contains information that causes the protein to be deposited in the spore coat; thus gene fusions that include some or all of a mature coat protein sequence are preferred for screening or selection for the display-of-IPBD phenotype.

Fusions of ipbd fragments to cotC or cotD fragments are likely to cause IPBD to appear on the spore surface. The genes cotC and cotD are preferred

osp genes because CotC and CotD are not post-translationally cleaved. Subsequences from cotA or cotB could also be used to cause an IPBD to appear on the surface of B. subtilis spores, but we must take the post-translational cleavage of these proteins into account. DNA encoding IPBD could be fused to a fragment of cotA or cotB at either end of the coding region or at sites interior to the coding region. Spores could then be screened or selected for the display-of-IPBD phenotype.

To date, no Bacillus sporulation promoter has been shown to be inducible by an exogenous chemical inducer as the lac promoter of E. coli. Nevertheless, the quantity of protein produced from a sporulation promoter can be controlled by other factors, such as the DNA sequence around the Shine-Dalgarno sequence or codon usage. Chemically inducible sporulation promoters can be developed if necessary.

Sec. 1.2.3: Choice of Insertion site for IPBD in OSP of Bacterial Spore:

The considerations governing insertion site in the spore OSP are the same as those given in Section 1.1.3.

Sec. 1.2.4: In Vivo Selection for Pseudo-osp Genes From Random DNA Inserts in Bacterial Spores:

Although the considerations for spores are nearly identical to the considerations for vegetative bacterial cells (Sec. 1.1), the available information on the mechanisms that cause proteins to appear on spores is meager so that use of the random-DNA approach becomes a more attractive option.

We can use the approach described above at 1.1.4 for attaching an IPBD to an E. coli cell, except that: a) a sporulation promoter is used, and b) no periplasmic signal sequence should be present.

Sec. 1.1: Displaying IPBD on Outer Surface of Phages:

Sec. 1.1.1: Preferred Phages for Use as OPs:

Unlike bacterial cells and spores, choice of a phage depends strongly on knowledge of the 3D structure of an OSP and how it interacts with other proteins in the capsid. The size of the phage genome and the packaging mechanism are also important because the phage genome itself is the cloning vector. The osp-ipbd gene must be inserted into the phage genome: therefore:

1) the virion must be capable of accepting the insertion or substitution of genetic material, and

2) the genome of the phage must be small enough to allow convenient manipulation.

Additional considerations in choosing phage are:

1) the morphogenetic pathway of the phage determines the environment in which the IPBD will have opportunity to fold,

2) IPBDs containing essential disulfides may not fold within a cell,

3) IPBDs needing large or insoluble prosthetic groups may not fold if secreted because the prosthetic group is lacking, and

4) when variegation is introduced in Part III, multiple infections could generate hybrid GPs that carry the gene for the PBD but have at least some copies of a different PBD on their surfaces; it is preferable to minimize this possibility.

Bacteriophages are excellent candidates for GPs because there is little or no enzymatic activity associated with intact mature phage, and because the genes are inactive outside a bacterial host, rendering the mature phage particles metabolically inert. The filamentous phage M13 and bacteriophage PhiX174 are of particular interest.

Filamentous phage :

The entire life cycle of the filamentous phage M13, a common cloning and sequencing vector, is well understood. M13 and f1 are so closely related that we consider the properties of each relevant to both (RASC86); any differentiation is for historical accuracy. The genetic structure (the complete sequence (SCHA78), the identity and function of the ten genes, and the order of transcription and location of the promoters) of M13 is well known as is the physical structure of the virion (BANN81, BOEK80, CHAN79, ITOK79, KAPL78, KUHN85b, KUHN87, MAKO80, MARV78, MESS78, OHKA81, RASC86, RUSS81, SCHA78, SMIT85, WEBS78, and ZIMM82); see RASC86 for a recent review of the structure and function of the coat proteins.

Filamentous phage enter E. coli through the sex pilus cells bearing the F-factor. Achtman et al. (ACHT78) observed that the pilus is extraordinarily sensitive to SDS; 0.03% SDS inhibits binding of MS2 to pilin in vitro. Infection may therefore be inhibited by SDS.

The 50 amino acid mature coat protein is synthesized as a 73 amino acid precoat (ITOK79). The first 23 amino acids constitute a typical signal-sequence which causes the nascent polypeptide to be inserted into the inner cell membrane.

An E. coli signal peptidase (SP-I) recognizes amino acids 18, 21, and 23, and, to a lesser extent, residue 22, and cuts between residues 23 and 24 of the precoat (KUHN85a, KUHN85b, OLIV87). (See also sec. 1.1.2 for general knowledge on secretion in E. coli.) After removal of the signal sequence, the amino terminus of the mature coat is located on the periplasmic side of the inner membrane; the carboxy terminus is on the cytoplasmic side. About 3000 copies of the mature 50 amino acid coat protein associate side-by-side in the inner membrane.

The gene VI, VII, and IX proteins are also present at the ends of the virion in about five copies each. The single-stranded circular phage DNA associates with about five copies of the gene III protein and is then extruded through the patch of membrane-associated coat protein in such a way that the DNA is encased in a helical sheath of protein (WEBS78). The DNA does not base pair (that would impose severe restrictions on the virus genome); rather the bases intercalate with each other independent of sequence. Because the M13 genome

is extruded through the membrane and coated by a large number of identical protein molecules, it can be used as a cloning vector (WATS87 p278, and MESS77). Thus we can insert extra genes into M13 and they will be carried along in a stable manner.

Marvin and collaborators (MARV78, MARO80, BANN81) have determined an approximate 3D virion structure of f1 by a combination of genetics, biochemistry, and X-ray diffraction from fibers of the virus. Figure 3 is drawn after the model of Banner et al. (BANN81) and shows only the C_{alpha}s of the protein. The apparent holes in the cylindrical sheath are actually filled by protein side groups so that the DNA within is protected. The amino terminus of each protein monomer is to the outside of the cylinder, while the carboxy terminus is at smaller radius, near the DNA. Although other filamentous phages (e.g. Pf1 or Ike) have different helical symmetry, all have coats composed of many short alpha-helical monomers with the amino terminus of each monomer on the virion surface.

Bacteriophage PhiX174 :

The bacteriophage PhiX174 is a very small icosahedral virus which has been thoroughly studied by genetics, biochemistry, and electron microscopy (See The Single-Stranded DNA Phages (DENH78)). To date, no proteins from PhiX174 have been studied by X-ray diffraction. PhiX174 is not used as a cloning vector because PhiX174 can accept almost no additional DNA: the virus is so tightly constrained that several of its genes overlap. Chambers et al. (CHAM82) showed that mutants in gene G are rescued by the wild-type G gene

carried on a plasmid so that the host supplies this protein.

Three gene products of PhiX174 are present on the outside of the mature virion: F (capsid), G (major spike protein, 60 copies per virion), and H (minor spike protein, 12 copies per virion). The G protein comprises 175 amino acids, while H comprises 328 amino acids. The F protein interacts with the single-stranded DNA of the virus. The proteins F, G, and H are translated from a single mRNA in the viral infected cells.

Large DNA Phages

Phage such as lambda or T4 have much larger genomes than do M13 or PhiX174. Large genomes are less conveniently manipulated than small genomes. A phage with a large genome, however, could be used if genetic manipulation is sufficiently convenient. Phage such as lambda and T4 have more complicated 3D capsid structures than M13 or PhiX174, with more OSPs to choose from. Phage lambda virions and phage T4 virions form intracellularly, so that IPBDs requiring large or insoluble prosthetic groups might fold on the surfaces of these phage.

RNA Phages

RNA phage, such as Qbeta, are not preferred because manipulation of RNA is much less convenient than is the manipulation of DNA. Although competent RNA bacteriophage are not preferred, useful genetically altered RNA-containing particles could be derived from RNA phage, such as MS2.

MS2 is a typical small RNA phage that carries only three genes that are tightly regulated through RNA structure and protein-RNA interactions. The RNA fills the protein capsid so that no additional genes can be accommodated. To use MS2 as a GP, we would need to eliminate most of the natural viral genome so that an osp-inbd gene could fit into the protein capsid. It is known that the A protein binds sequence-specifically to a site at the 5' end of the + RNA strand triggering formation of RNA-containing particles if coat protein is present. If a message containing the A protein binding site and the gene for a chimera of coat protein and a PBD were produced in a cell that also contained A protein and wild-type coat protein (both produced from regulated genes on a plasmid), then the RNA coding for the chimeric protein would get packaged. The viral RNA replicase gene is not needed because all components needed for formation of particles are encoded in DNA. A package comprising RNA encapsulated by proteins encoded by that RNA satisfies the major criterion that the genetic message inside the package specifies something on the outside. The particles by themselves are not viable. After isolating the packages that carry an SBD, we would need to:

1. separate the RNA from the protein capsid,

2) reverse transcribe the RNA into DNA, using AMV or MMTV reverse transcriptase, and

3) use Thermus aquaticus DNA polymerase for 25 or more cycles of Polymerase Chain Reaction(TM) to amplify the DNA until there is enough to subclone

the recovered genetic message into a plasmid for sequencing and further work.

Alternatively, helper phage could be used to rescue the isolated phage. In one of these ways we can recover a sequence that codes for an SBD having desirable binding properties. The in vitro amplification (SAIK85, SCHA86, US Patents 4,683,202 and 4,683,195) may be conveniently carried out using a Perkin-Elmer/Cetus Thermal Cycler (part number N801-0150) and GeneAmp DNA Amplification Reagent Kit (N801-0043) supplied by Perkin-Elmer Corp., 761 Main Avenue, Norwalk, CT, 06859-0012, USA. The primers used in the Polymerase Chain Reaction(TM) should be picked so that the osp-pbd gene is the part of the reverse-transformed DNA that is amplified.

Although such a procedure is much more cumbersome than use of DNA phage, it may be of interest if: 1) the genetic package of the RNA phage is much more stable than any DNA phage, 2) the 3D structure of an RNA phage is known (f2 forms crystals inside E. coli, suggesting that structure determination of f2 virion may be practical), or 3) folding of a large protein inside a cell is desired (this scheme allows almost the entire 3.5 Kb genome of MS2 to be used for chimeric coat protein-PBD). Use of fusions involving MS2 coat protein, together with wild-type MS2 coat protein, to encapsulate genes demonstrates the most primitive system that could be employed in the present invention. Although the system has certain technical inconveniences and therefore is not preferred, it could be used.

Sec. 1.3.2: Preferred Outer-Surface Proteins for Displaying IPBDs on Phages:

For a given bacteriophage, the preferred OSP is usually one that is present on the phage surface in the largest number of copies, as this allows the greatest flexibility in varying the ratio of OSP-IPBD to wild type OSP and also gives the highest likelihood of obtaining satisfactory affinity separation. Moreover, a protein present in only one or a few copies usually performs an essential function in morphogenesis or infection; mutating such a protein by addition or insertion is likely to result in reduction in viability of the GP.

It is preferred that the wild-type *osp* gene be preserved. The *ipbd* gene fragment may be inserted either into a second copy of the recipient *osp* gene or into a novel engineered *osp* gene. It is preferred that the *osp-ipbd* gene be placed under control of a regulated promoter. Our process forces the evolution of the PBDs derived from IPBD so that some of them develop a novel function, *viz.* binding to a chosen target. Placing the gene that is subject to evolution on a duplicate gene is an imitation of the widely-accepted scenario for the evolution of protein families. It is now generally accepted that gene duplication is the first step in the evolution of one protein from an ancestral protein. By having two copies of a gene, the affected physiological process can tolerate mutations in one of the genes. This process is well understood and documented for the globin family (*cf.* DICK83, p65ff, and CREI84, p117-125).

The preferred OSP for use when the GP is M13 is the gene III protein (see Example 1).

Sec. 1.3.3: Choice of Insertion site for IPBD in OSP:

The user must choose a site in the candidate OSP gene for inserting a ipbd gene fragment. The coats of most bacteriophage are highly ordered. Filamentous phage can be described by a helical lattice; isometric phage, by an icosahedral lattice. Each monomer of each major coat protein sits on a lattice point and makes defined interactions with each of its neighbors. Proteins that fit into the lattice by making some, but not all, of the normal lattice contacts are likely to destabilize the virion by: a) aborting formation of the virion, b) making the virion unstable, or c) leaving gaps in the virion so that the nucleic acid is not protected. Thus in bacteriophage, unlike the cases of bacteria and spores, it is important to retain most or all of the residues of the parental OSP in engineered OSP-IPBD fusion proteins.

Association of proteins into dimers, trimers, or even larger structures represents yet another aspect of protein binding. For proteins that form such associations, heterologous mixtures of mutant and normal proteins will form if the mutations have not altered the interface between subunits. For example, Ward et al. have shown that tyrosyl tRNA synthetase will form heterodimers when mutant and normal protein are allowed to refold together (WARD86). See also Hickman and Levy (HICK88) who studied the multimeric structures of the Tet^R protein by engineering cells to carry two different tet alleles and observing a Tet^R phenotype arising from the complementary alleles. They

conclude that the Tet^R protein is multimeric.

Immunoglobulin formation depends on the ability of V_L domains and V_H domains, each a part of a separately synthesized protein, to associate independently of the protein sequence in the antigen complementarity-determining regions. In addition, the process of immune complementation depends on the separability of the binding properties of the complementarity-determining regions from the binding properties of the constant domains.

Auditore-Hargreaves, US Patents 4,470,925 (AUDI84a) and 4,479,895 (AUDI84b) teaches methods of making hybrid antibodies that depend on association of different antibody chains. These patents teach that alterations far from the intermolecular interface do not alter the association.

A preferred site for insertion of the ipbd gene into the phage osp gene is one in which: a) the IPBD folds into its original shape, b) the OSP domains fold into their original shapes, and c) there is no interference between the two domains. It is not required that the IPBD and OSP domains have any particular spatial relationship; hence the process of this invention does not require use of the method of US Patent '692.

If there is a 3D model of the phage that indicates that either the amino or carboxy terminus of an OSP is exposed to solvent, then the exposed terminus of that mature OSP becomes the prime candidate for insertion of the ipbd gene. A low resolution 3D model suffices.

In the absence of a 3D structure, the amino and carboxy termini of the mature OSP are the best candidates for insertion of the ipbd gene. A functional fusion may require additional residues between the IPBD and OSP domains to avoid unwanted interactions between the domains. Random-sequence DNA or DNA coding for a specific sequence of a protein homologous to the IPBD or OSP, can be inserted between the osp fragment and the ipbd fragment if needed.

Fusion at a domain boundary within the OSP is also a good approach for obtaining a functional fusion. Smith exploited such a boundary when subcloning heterologous DNA into gene III of f1 (SMIT85).

There are several methods of identifying domains. Methods that rely on atomic coordinates have been reviewed by Janin and Chothia (JANI85). These methods use matrices of distances between alpha carbons (C_{alpha}), dividing planes (cf. ROSE85), or buried surface (RASH84). Chothia and collaborators have correlated the behavior of many natural proteins with domain structure (according to their definition). Rashin correctly predicted the stability of a domain comprising residues 206-316 of thermolysin (VITA84, RASH84).

Many researchers have used partial proteolysis and protein sequence analysis to isolate and identify stable domains. (See, for example, VITA84, POTE81, SCOT87, and PABO79.) Pabo et al. used calorimetry as an indicator that the cI repressor from the coliphage lambda contains two domains; they then used partial proteolysis to determine the location of the domain boundary.

It is generally believed that the part of the polypeptide chain composing one domain folds almost independently of the parts composing other domains. There are natural proteins composed of two or more domains for which there is strong evidence that essentially the same domain occurs more than once, for example ovomucoids and ovoinhibitors (SCOT87) and kallikrein (CHUN86). Further, the same domain can occur in several different proteins (SUDH85, GILB85, and SCOT87).

If the only structural information available is the amino acid sequence of the candidate OSP, we can use the sequence to predict turns and loops. There is a high probability that some of the loops and turns will be correctly predicted (cf. Chou and Fasman, (CHOU72)): these locations are also candidates for insertion of the ipbd gene fragment.

Sec. 1.3.4: In Vivo Selection for Pseudo-OSP Gene from Random DNA Inserts in Bacterial Spores:

Alternatively, a functional insertion site may be determined by generating a number of recombinant constructions and selecting the functional strain by phenotypic characteristics. Because the OSP-IPBD must fulfill a structural role in the phage coat, it is unlikely that any particular random DNA sequence coupled to the ipbd gene will produce a fusion protein that fits into the coat in a functional way. Nevertheless, random DNA inserted between large fragments of a coat protein gene and the ipbd gene will produce a population that is likely to contain one or more members that display the IPBD on the outside of a

viable phage. A display probe, similar to that defined in 1.1.4, is constructed and random DNA sequences cloned into appropriate sites.

Sec. 2: Choice of IPBD :

A IPBD may be chosen from naturally occurring proteins or domains of naturally occurring proteins, or may be designed from first principles. A designed protein may have advantages over natural proteins if: a) the designed protein is more stable, b) the designed protein is smaller, and c) the charge distribution of the designed protein can be specified more freely.

A candidate IPBD must meet the following criteria:

1) a domain exists that will remain stable under the conditions of its intended use (the domain may comprise the entire protein that will be inserted, e.g. BPTI),

2) knowledge of the amino acid sequence is obtainable,

3) knowledge of the identity of the residues on the domain's outer surface, and their spatial relationships, is obtainable, and

4) a molecule is available having specific and high affinity for the IPBD, AfM(IPBD).

Preferably, the IPBD is no larger than necessary because it is easier to arrange restriction sites in

smaller amino-acid sequences and because a smaller protein minimizes the metabolic strain on the GP or the host of the GP. The usefulness of candidate IPBDs that meet all of these requirements depends on the availability of the information discussed below.

Information about candidate IPBDs that will be used to judge the suitability of the IPBD includes: 1) a 3D structure (knowledge strongly preferred), 2) one or more sequences homologous to the IPBD (the more homologous sequences known, the better), 3) the pI of the IPBD (knowledge necessary in some cases), 4) the stability and solubility as a function of temperature, pH and ionic strength (preferably known to be stable over a wide range and soluble in conditions of intended use), 5) ability to bind metal ions such as Ca^{++} or Mg^{++} (knowledge preferred: binding per se, no preference), 6) enzymatic activities, if any (knowledge preferred, activity per se has uses but may cause problems), 7) binding properties, if any (knowledge preferred, specific binding also preferred), 8) availability of a molecule having specific and strong affinity ($K_d < 10^{-11}$ M) for the IPBD (preferred), 9) availability of a molecule having specific and medium affinity (10^{-8} M $< K_d < 10^{-6}$ M) for the IPBD (preferred), 10) the sequence of a mutant of IPBD that does not bind to the affinity molecule(s) (preferred), and 11) absorption spectrum in visible, UV, NMR, etc. (characteristic absorption preferred).

If only one species of molecule having affinity for IPBD (AfM(IPBD)) is available, it will be used to: a) detect the IPBD on the GP surface, b) optimize expression level and density of the affinity molecule on the matrix (Sec. 10.1), and c) determine the

efficiency and sensitivity of the affinity separation (Secs. 10.2 and 10.3). As noted above, however, one would prefer to have available two species of AfN(IPBD), one with high and one with moderate affinity for the IPBD. The species with high affinity would be used in initial detection and in determining efficiency and sensitivity (10.2 and 10.3), and the species with moderate affinity would be used in optimization (10.1).

There are many candidate IPBDs, 20 or more, for which all of the above information is available or is reasonably practical to obtain, for example, bovine pancreatic trypsin inhibitor (BPTI, 58 residues), crambin (46 residues), third domain of ovomucoid (56 residues), T4 lysozyme (164 residues), and azurin (128 residues). Structural information can be obtained from X-ray or neutron diffraction studies, NMR, chemical cross linking or labeling, modeling from known structures of related proteins, or from theoretical calculations. 3D structural information obtained by X-ray diffraction, neutron diffraction or NMR is preferred because these methods allow localization of almost all of the atoms to within defined limits.

Most of the PBDs derived from a PPBD according to the process of the present invention affect residues having side groups directed toward the solvent. Reidhaar-Olson and Sauer (REIDSS) found that exposed residues can accept a wide range of amino acids, while buried residues are more limited in this regard. Surface mutations typically have only small effects on melting temperature of the PBD, but may reduce the stability of the PBD. Hence the chosen IPBD should have a high melting temperature (60°C acceptable, the higher the better) and be stable over a wide pH range

(8.0 to 3.0 acceptable; 11.0 to 2.0 preferred), so that the SBDs derived from the chosen IPBD by mutation and selection-through-binding will retain sufficient stability. Preferably, the substitutions in the IPBD yielding the various PBDs do not reduce the melting point of the domain below 50°C. Mutations may arise that increase the stability of SBDs relative to the IPBD, but the process of the present invention does not depend upon this occurring.

Two general characteristics of the target molecule, size and charge, make certain classes of IPBDs more likely than other classes to yield derivatives that will bind specifically to the target. Because these are very general characteristics, one can divide all targets into six classes: a) large positive, b) large neutral, c) large negative, d) small positive, e) small neutral, and f) small negative. A small collection of IPBDs, one or a few corresponding to each class of target, will contain a preferred candidate IPBD for any chosen target.

Alternatively, the user may elect to engineer a GP(IPBD) for a particular target; Sec 2.1 gives criteria that relate target size and charge to the choice of IPBD.

Sec. 2.1.1: Influence of target size on choice of IPBD:

If the target is a protein or other macromolecule a preferred embodiment of the IPBD is a small protein such as BPTI from Bos Taurus (58 residues), crambin from rape seed (46 residues), or the third domain of ovomucoid from Coturnix coturnix Japonica (Japanese quail) (56 residues) (PAPA82), because targets from

this class have clefts and grooves that can accommodate small proteins in highly specific ways. If the target is a macromolecule lacking a compact structure, such as starch, it should be treated as if it were a small molecule. Extended macromolecules with defined 3D structure, such as collagen, should be treated as large molecules.

If the target is a small molecule, such as a steroid, a preferred embodiment of the IPBD is a protein the size of ribonuclease from Bos taurus (124 residues), ribonuclease from Aspergillus oryzae (104 residues), hen egg white lysozyme from Gallus gallus (129 residues), azurin from Pseudomonas aeruginosa (128 residues), or T4 lysozyme (164 residues), because such proteins have clefts and grooves into which the small target molecules can fit. The Brookhaven Protein Data Bank contains 3D structures for all of the proteins listed. Genes encoding proteins as large as T4 lysozyme can be manipulated by standard techniques for the purposes of this invention.

If the target is a mineral, insoluble in water, one must consider the nature of the molecular surface of the mineral. Minerals that have smooth surfaces, such as crystalline silicon, require medium to large proteins, such as ribonuclease, as IPBD in order to have sufficient contact area and specificity. Minerals with rough, grooved surfaces, such as zeolites, could be bound either by small proteins, such as BPTI, or larger proteins, such as T4 lysozyme.

Sec. 2.1.2: Influence of target charge on choice of IPBD:

Electrostatic repulsion between molecules of like charge can prevent molecules with highly complementary surfaces from binding. Therefore, it is preferred that, under the conditions of intended use, the IPBD and the target molecule either have opposite charge or that one of them is neutral. In some cases it has been observed that protein molecules bind in such a way that like charged groups are juxtaposed by including oppositely charged counter ions in the molecular interface. Thus, inclusion of counter ions can reduce or eliminate electrostatic repulsion and the user may elect to include ions in the eluants used in the affinity separation step. Polyvalent ions are more effective at reducing repulsion than monovalent ions.

Sec. 2.1.3: Other considerations in the choice of IPBD:

If the chosen IPBD is an enzyme, it may be necessary to change one or more residues in the active site to inactivate enzyme function. For example, if the IPBD were T4 lysozyme and the GP were E. coli cells or M13, we would need to inactivate the lysozyme because otherwise it would lyse the cells. If, on the other hand, the GP were PhiX174, then inactivation of lysozyme may not be needed because T4 lysozyme can be overproduced inside E. coli cells without detrimental effects and PhiX174 forms intracellularly. It is preferred to inactivate enzyme IPBDs that might be harmful to the GP or its host by substituting mutant amino acids at one or more residues of the active site. It is permitted to vary one or more of the residues that were changed to abolish the original enzymatic activity of the IPBD. Those GPs that receive osp-cbd

genes encoding an active enzyme may die, but the majority of sequences will not be deleterious.

If the binding protein is intended for therapeutic use in humans or animals, the IPBD may be chosen from proteins native to the designated recipient to minimize the possibility of antigenic reactions.

Sec. 3: Choice of OCV :

The OCV is preferably small, e.g., less than 10 KB. The size of the OCV affects the stability of the OCV and its derivatives, and the copy number thereof. An OCV which is stable, even after insertion of at least 1 kb DNA, is sought. A multicopy OCV is also of interest. It is desirable that cassette mutagenesis be practical in the OCV; preferably, at least 25 restriction enzymes are available that do not cut the OCV. It is likewise desirable that single-stranded mutagenesis be practical. Finally, the OCV preferably carriers a selectable marker.

If a suitable OCV does not already exist, it may be engineered by manipulation of available vectors.

In the cases of bacterial cells and bacterial spores, the bacterial chromosome could be used as the OCV. Plasmids are, however, preferred because genes on plasmids are much more easily constructed and mutated than are genes in the bacterial chromosome. When bacteriophage are to be used, the esp-ipbd gene must be inserted into the phage genome. The synthetic esp-ipbd genes can be constructed in small vectors and transferred to the GP genome when complete.

Phage such as M13 do not confer antibiotic resistance on the host so that one can not select for cells infected with M13. An antibiotic resistance gene can be engineered into the M13 genome (HINE80). More virulent phage, such as PhiX174, make discernable plaques that can be picked, in which case a resistance gene is not essential: furthermore, there is no room in the PhiX174 virion to add any new genetic material. Inability to include an antibiotic resistance gene is a disadvantage because it limits the number of GPs that can be screened.

It is preferred that GP(IPBD) carry a selectable marker not carried by wtGP. It is also preferred that wtGP carry a selectable marker not carried by GP(IPBD).

Sec. 4: Designing the osp-ipbd gene insert:

Having chosen a IPBD, a GP, a strategy for getting the IPBD onto the GP surface, and a cloning vector, we now turn to the design of a suitably regulated gene. In this section, we design an amino acid sequence that will cause the IPBD to appear on the GP surface when it is expressed. This amino acid sequence may determine the entire coding region of the osp-ipbd gene, or it may contain only the ipbd sequence adjoining restriction sites into which random DNA will be cloned (Sec. 6.2).

We will now consider the transcriptional regulation of the osp-ipbd gene: the design of the DNA encoding of amino acid sequences: the organization of synthesis; the methods of DNA synthesis and purification; and the actual gene synthesis and cloning.

The actual gene may be: a) completely synthetic, b) a composite of natural and synthetic DNA, or c) a composite of natural DNA fragments. The important point is that the pbd segment, derived from the ipbd segment, be easily genetically manipulated in the ways described in Part III. A synthetic ipbd segment is preferred because it allows greatest control over placement of restriction sites. Primers complementary to regions abutting the osp-ipbd gene on its 3' flank and to parts of the osp-ipbd gene that are not to be varied are needed for sequencing.

Sec. 4.1 Genetic regulation of the osp-ipbd gene:

Now we consider regulation of the osp-ipbd gene to enable modulation of expression. The two important questions are: a) how much OSP-IPBD do we need on each GP, and b) how accurately must we regulate the amount?

The essential function of the affinity separation is to separate GPs that bear PBDs (derived from IPBD) having high affinity for the target from GPs bearing PBDs having low affinity for the target. If the elution volume of a GP depends on the number of PBDs on the GP surface, then a GP bearing many PBDs with low affinity, GP(PBD_w), might co-elute with a GP bearing fewer PBDs with high affinity, GP(PBD_s). Assume that both GP(PBD_w) and GP(PBD_s) bind to the column under some condition, such as low salt. If a gradient of some solute, such as increasing salt, changes the conditions, then all weakly-binding PBDs will cease to bind before any strongly-binding PBDs cease to bind. Regulation of the osp-pbd gene must be such that all packages display sufficient PBD to effect a good

separation in Sec 15. If the amount of PBD/GP had an effect on the elution volume of the GP from the affinity matrix, then we would need to regulate the amount of PBD/GP very accurately. The following analysis shows that there is no strong linear effect of IPBD/GP on elution volume and assumes only: a) that all GPs are the same size, b) that interactions between the PBDs and the affinity matrix dominate differential elution of GPs, c) that the system is at equilibrium, and d) that all PBDs on any one GP are identical.

If N_p identical PBDs on a GP each have access to target molecules, and each PBD has a free-energy of binding to the target of delta G_b, then the total free energy of binding is

$$\text{delta } G_b^{tot} = N_p * \text{delta } G_b .$$

Delta G_b will be a function of several parameters of the solvent, such as: 1) concentration of ions, 2) pH, 3) temperature, 4) concentration of neutral solutes such as sucrose, glucose, ethanol, etc., 5) specific ions, such as, calcium, acetate, benzoate, nicotinate, etc. If conditions are altered during affinity separation so that delta G_b approaches zero, delta G_b^{tot} approaches zero Np times faster. As delta G_b^{tot} goes to or above zero, the packages will dissociate from the immobilized target molecules and be eluted.

GPs bearing more PBDs have a sharper transition between bound and unbound than packages with fewer of the same PBDs. For equilibrium conditions, the mid-point of the transition is determined only by the solution conditions that bring the individual

interactions to zero free-energy. The number of PBDs/GP determines the sharpness of the transition.

It should also be noted that the number of PBDs/GP is usually influenced by physiological conditions so that a sample of genetically identical GP(PBD)s may contain GPs having different numbers of PBDs on the GP surface. In a population of GP(vgPBD)s each PBD sequence will appear on more that one GP, and the actual number of PBDs/GP will vary from GP to GP within some range. Within a variegated population of PBDs, let PBD_X be the PBD with maximum affinity for the target. If there is a linear effect of number of PBDs/GP, then the GPs having the greatest number of PBD_X will be most retarded on the column. When we culture the enriched population obtained either as an effluent from the column or as an inoculum of matrix material from the column, the GP(PBD_X) will be amplified and give rise to new GP(PBD_X)s having varying numbers of PBD_X/GP. Thus the affinity separation process of the present invention could tolerate a linear effect of number of PBDs/GP on the elution volume of the GP(PBD) unless strong binding to target fortuitously causes the PBD to be displayed on the GP only in low number. It is extremely unlikely that all PBDs that bind to the target will also be incapable of display in large amounts on the GP surface.

According to the above analysis, there is no linear effect on elution volume from the number of IPBDs/GP, hence need for highly accurate regulation of IPBD/GP is not anticipated. The analysis above assumes that GP(IPBD)s are in equilibrium between solution in buffer and bound to the affinity matrix. Rate of elution may be an important parameter in column

affinity chromatography. In batch elution from an affinity matrix or elution from an affinity plate, the time that each buffer is in contact with the affinity material may be an important variable. The density of affinity molecules on the matrix is an important variable in optimizing the affinity separation. Because the analysis above is qualitative, in Sec. 10 of the preferred embodiment we experimentally optimize: 1) the density of IPBD on the GP surface, 2) the density of affinity molecules on the affinity matrix, 3) the initial ionic strength, 4) the elution rate, and 5) the quantity of GP/(volume of matrix) to be loaded on the column.

A number of promoters are known that can be controlled by specific chemicals added to the culture medium. For example, the lacUV5 promoter is induced if isopropylthiogalactoside is added to the culture medium, for example, at between 1.0 uM and 10.0 mM. Hereinafter, we use "XINDUCE" as a generic term for a chemical that induces expression of a gene.

Transcriptional regulation of gene expression is best understood and most effective, so we focus our attention on the promoter. If transcription of the osp-ipbd gene is controlled by the chemical XINDUCE, then the number of OSP-IPBDs per GP increases for increasing concentrations of XINDUCE until a fall-off in the number of viable packages is observed or until sufficient IPBD is observed on the surface of harvested GP(IPBD)s. The attributes that affect the maximum number of OSP-IPBDs per GP are primarily structural in nature. There may be steric hindrance or other unwanted interactions between IPBDs if OSP-IPBD is substituted for every wild-type OSP. Excessive levels

of OSP-IPBD may also adversely affect the solubility or morphogenesis of the GP. For cellular and viral GPs, as few as five copies of a protein having affinity for another immobilized molecule have resulted in successful affinity separations (FERE82a, FERE82b, and SMIT85).

Another consideration of promoter regulation is that it is useful later to know the range of regulation of the osp-ipbd. (Sec. 8) In particular, one should determine how nearly the absence of XINDUCE leads to the absence of IPBD on the GP surface; a non-leaky promoter is preferred. Non-leakiness is useful: a) to show that affinity of GP(osp-ipbd)s for AfM(IPBD) is due to the osp-ipbd gene, and b) to allow growth of GP(osp-pbd) in the absence of XINDUCE if the expression of osp-pbd is disadvantageous. The lacUV5 promoter in conjunction with the LacI^q repressor is a preferred example.

Sec. 4.2: DNA sequence design:

The present invention is not limited to a single method of gene design. The following procedure is an example of one method of gene design that fills the needs of the present invention.

Having specified that the amount of IPBD/GP is to be experimentally optimized and that well-studied available regulatory mechanisms applied to osp-ipbd gene are sufficient, we now consider design of a DNA sequence. If the amino-acid sequence of OSP-IPBD is a definite sequence, then the entire gene will be constructed (Sec. 6.1). If random DNA is to be fused to ipbd, then a "display probe" is constructed first;

the random DNA is then inserted to complete the population of putative osp-ipbd genes (Sec. 6.2) from which a functional osp-ipbd gene is identified by in vivo selection or kindred techniques.

The osp-ipbd gene need not be synthesized in toto; parts of the gene may be obtained from nature. One may use any genetic engineering method to produce the correct gene fusion, so long as one can easily and accurately direct mutations to specific sites in the pbd DNA subsequence (Sec. 14.1). In all of the methods of mutagenesis considered in the present invention, however, it is necessary that the DNA sequence for the osp-ipbd gene be different from any other DNA in the OCV. The degree and nature of difference needed is determined by the method of mutagenesis to be used in Sec. 14.1. If the method of mutagenesis is to be replacement of subsequences coding for the PBD with vgDNA, then the subsequences to be mutagenized must be bounded by restriction sites that are unique with respect to the rest of the OCV. If single-stranded-oligonucleotide-directed mutagenesis is to be used, then the DNA sequence of the subsequence coding for the IPBD must be unique with respect to the rest of the OCV.

The sequences of regulatory parts of the gene are taken from the sequences of natural regulatory elements: a) promoters, b) Shine-Dalgarno sequences, and c) transcriptional terminators. Regulatory elements could also be designed from knowledge of consensus sequences of natural regulatory regions. The sequences of these regulatory elements are connected to the coding regions; restriction sites are also inserted

in or adjacent to the regulatory regions to allow convenient manipulation.

The coding portions of genes to be synthesized are designed at the protein level and then encoded in DNA. The amino acid sequences are chosen to achieve various goals, including: a) display of a IPBD on the surface of a GP, b) change of charge on a IPBD, and c) generation of a population of PBDs from which to select an SBD. The ambiguity in the genetic code is exploited to allow optimal placement of restriction sites and to create various distributions of amino acids at variegated codons.

Sec. 4.1: Specific DNA sequence assignment:

A computer program may be used to construct an ambiguous DNA sequence coding for an amino-acid sequence given by the user. That is, the DNA sequence contains codes for all possible DNA sequences that produce the stated amino acid sequence. The codes used in the ambiguous DNA are shown in Table 1. An example of an ambiguous DNA sequence is given in Table 3.

The user supplies lists of restriction enzymes that: a) do not cut the OCV, and b) cut the OCV only once or twice. For each enzyme the program reads: a) the name, b) the recognition sequence, c) the cutting pattern, and d) the names of suppliers. The ambiguous DNA sequence coding for the stated amino acid sequence is examined for places that recognition sites for any of the given enzymes could be created without altering the amino-acid sequence. A master table of enzymes could be obtained from the catalogues of enzyme suppliers such as the suppliers listed in Table 4 or

other sources, such as Roberts' annual review of restriction enzymes in Nucleic Acids Research.

Each potential recognition site causes a record similar to the following to be written:

```
Hind III<S,B,M,I,N,P> Loc=9 T=9 B=13 Dir=n Cut elective
Protein seq   : k - s - l - w
   aa #s      : 3 |  4 |  5 |  6      Cut # 1/6
possible DNA :AAr|AGy|TTr|TGG 5'NNNNNA      AGCTTNNNNN3'
cutter       :  A|AGC|TT       3'NNNNNTTCGA      ANNNNN5'
result       :AAA|AGC|TTr
```

The top line identifies the enzyme, Hind III in this example, and the supplier (through codes given in Table 4); "Loc=9" indicates that recognition begins with nucleotide 9; "T=9" indicates that the antisense (top) strand of DNA is cut after base 9; "B=13" indicates that the sense strand (or bottom strand, not shown except in the dsDNA on the right) is cut between bases 13 and 14 (reading left to right). "Dir=n" indicates that recognition is "normal". Hind III recognizes palindromic sequences, as do most restriction enzymes. Some enzymes have asymmetric recognition, however, and cut to one side; for these enzymes, the recognition could be "normal" or "reversed" depending on whether the enzyme cuts to the right or left of the recognition site. Rare unambiguous stretches that require certain restriction sites are labeled as "obligatory"; those that are elective are so labeled.

The second and third lines show the amino-acid sequence and residue numbers for which this region of DNA codes. The notation "Cut # 1/6" indicates that this is the first of six possible Hind III sites.

The fourth line shows the antisense strand of DNA coding for the desired amino-acid sequence. The fifth line shows the recognition pattern of the enzyme. The sixth line shows the consensus between the DNA sequence required by the amino acid sequence and the DNA sequence recognized by the restriction enzyme. The dsDNA to the right shows the ends generated by the restriction digestion.

The program also prints a table summarizing the possible sites. An example of such a summary of potential sites is found in Table 5.

The choice of elective restriction sites to be built into the gene is determined as follows.

The goal is to have a series of fairly uniformly spaced unique restriction sites with no more than a preset maximum number of bases, for example 100, between sites. Unless required by other sites, sites that are not present in the parental OCV are not introduced into the designed gene more than once. Sites that occur only once or twice in the parental OCV are not introduced into the designed gene unless necessary.

First, each enzyme that has a unique possible site is picked; if two of these overlap, then the better enzyme is picked. An enzyme is better if it: a) generates cohesive ends, b) has unambiguous recognition, or c) has higher specific activity. Next, those sites close to other sites already picked are eliminated because many sites very close together are not useful. Finally, sites are chosen to minimize the size of the longest piece between restriction sites.

The ambiguity of the DNA between the restriction sites is resolved from the following considerations. If the given amino acid sequence occurs in the recipient organism, and if the DNA sequence of the gene in the organism is known, then, preferably, we maximize the differences between the engineered and natural genes to minimize the potential for recombination. In addition, the following codons are poorly translated in E. coli and, therefore, are avoided if possible: cta(L), cga (R), cgg (R), and agg (R). For other host species, different codon restrictions would be appropriate. Finally, long repeats of any one base are prone to mutation and thus are avoided. Balancing these considerations, we can design a DNA sequence.

Sec. 5.1: Organization of gene synthesis:

Now we consider ways to divide the synthesis of the designed gene into manageable segments. The present invention is not limited as to how a designed DNA sequence is divided for easy synthesis. The following procedure is an example of how such synthesis might be managed.

An established method is to synthesize both strands of the entire gene in overlapping segments of 20 to 50 nucleotides (nts) (THER83). Below we provide an alternative method that is more suitable for synthesis of vgDNA. This method is similar to methods published by Oliphant et al. (OLIP86 and OLIP87) and Ausubel et al. (AUSU87). Our adaptation of this method differs from previous methods in that we: a) use two synthetic strands, and b) do not cut the extended DNA in the middle. Our goals are: a) to produce longer

pieces of dsDNA than can be synthesized as ssDNA on commercial DNA synthesizers, and b) to produce strands complementary to single-stranded vgDNA. By using two synthetic strands, we remove the requirement for a palindromic sequence at the 3' end.

DNA synthesizers can currently produce oligo-nts of lengths up to 100 nts in reasonable yield, M_{DNA} = 100. The parameters N_w (the length of overlap needed to obtain efficient annealing) and N_s (the number of spacer bases needed so that a restriction enzyme can cut near the end of blunt-ended dsDNA) are determined by DNA and enzyme chemistry. N_w = 10 and N_s = 5 are reasonable values. Larger values of N_w and N_s are allowed but add to the length of ssDNA that must be synthesized and reduce the net length of dsDNA that can be produced.

Let A_L be the actual length of dsDNA to be synthesized, including any spacers. A_L must be no greater than $(2\ M_{DNA} - N_w)$. Let Q_w be the number of nts that the overlap window can deviate from center,

$$Q_w = (2\ M_{DNA} - N_w - A_L)/2 .$$

Q_w is never negative. It is preferred that the two fragments be approximately the same length so that the amounts synthesized will be approximately equal. This preference may be overridden by other considerations. The overall yield of dsDNA is usually dominated by the synthetic yield of the longer oligo-nt.

We use the following procedure to generate dsDNA of lengths up to $(2\ M_{DNA} - N_w)$ nts through the use of

Klenow fragment to extend synthetic ss DNA fragments that are not more than M_{DNA} nts long. When a pair of long oligo-nts, complementary for N_w nts at their 3' ends, are annealed there will be a free 3' hydroxyl and a long ssDNA chain continuing in the 5' direction on either side. We will refer to this situation as a 5' superoverhang. The procedure comprises:

1) picking a non-palindromic subsequence of N_w to N_w+4 nts near the center of the dsDNA to be synthesized; this region is called the overlap (typically, N_w is 10),

2) synthesizing a ss DNA molecule that comprises that part of the anti-sense strand from its 5' end up to and including the overlap,

3) synthesizing a ss DNA molecule that comprises that part of the sense strand from its 5' end up to and including the overlap,

4) annealing the two synthetic strands that are complementary throughout the overlap region, and

5) extending both superoverhangs with Klenow fragment and all four deoxynucleotide triphosphates.

Because M_{DNA} is not rigidly fixed at 100, the current limits of 190 (= 2 M_{DNA} - N_w) nts overall and 100 in each fragment are not rigid, but can be exceeded by 5 or 10 nts. Going beyond the limits of 190 and 100 will lead to lower yields, but these may be acceptable in certain cases.

Restriction enzymes do not cut well at sites closer than about five base pairs from the end of blunt ds DNA fragments (OLIP87). Therefore N_S nts (with N_S typically set to 5) of spacer are added to ends that we intend to cut with a restriction enzyme. If the plasmid is to be cut with a blunt-cutting enzyme, then we do not add any spacer to the corresponding end of the ds DNA fragment.

To choose the optimum site of overlap for the oligo-nt fragments, first consider the anti-sense strand of the DNA to be synthesized, including any spacers at the ends, written (in upper case) from 5' to 3' and left-to-right. N.B.: The N_W nt long overlap window can never include bases that are to be variegated. N.B.: The N_W nt long overlap should not be palindromic lest single DNA molecules prime themselves. Place a N_W nt long window as close to the center of the anti-sense sequence as possible. Check to see whether one or more codons within the window can be changed to increase the GC content without: a) destroying a needed restriction site, b) changing amino acid sequence, or c) making the overlap region palindromic. If possible, change some AT base pairs to GC pairs. If the GC content of the window is less than 50%, slide the window right or left as much as Q_W nts to maximize the number of C's and G's inside the window, but without including any variegated bases. For each trial setting of the overlap window, maximize the GC content by silent codon changes, but do not destroy wanted restriction sites or make the overlap palindromic. If the best setting still has less than 50% GC, enlarge the window to N_W+2 nts and place it within five nts of the center to obtain the maximum GC content. If

enlarging the window one or two nts will increase the GC content, do so, but do not include variegated bases.

Underscore the anti-sense strand from the 5' end up to the right edge of the window. Write the complementary sense sequence 3'-to-5' and left-to-right and in lower case letters, under the anti-sense strand starting at the left edge of the window and continuing all the way to the right end of the anti-sense strand.

We will synthesize the underscored anti-sense strand and the part of the sense strand that we wrote. These two fragments, complementary over the length of the window of high GC content, are mixed in equimolar quantities and annealed. These fragments are extended with Klenow fragment and all four deoxynucleotide triphosphates to produce ds blunt-ended DNA. This DNA can be cut with appropriate restriction enzymes to produce the cohesive ends needed to ligate the fragment to other DNA.

Sec. 5.2: DNA synthesis and purification methods :

The present invention is not limited to any particular method of DNA synthesis or construction. The following procedures exemplify one way to achieve the goals of the present invention.

DNA is synthesized on a Milligen 7500 DNA synthesizer (Milligen, a division of Millipore Corporation, Bedford, MA) by standard procedures. Software to control the synthesizer and to keep records of each synthesis is supplied by Milligen.

The following reagents are supplied by Milligen:

1) 2.5% 1H-tetrazole in acetonitrile,
2) 3% (v/v) dichloroacetic acid in dichloromethane,
3) Acetic anhydride in 2,6 lutidine/acetonitrile (1:1:8),
4) 6.5% dimethylaminopyridine in acetonitrile,
5) 0.1M iodine in 2,6 lutidine/water/tetrahydrofuran (8:8:84),
6) 3% (v/v) triethylamine in acetonitrile,
7) DMT-dAdenosine(Bz)cyanoethylphosphoramidite
8) DMT-dCytidine(Bz)cyanoethylphosphoramidite
9) DMT-dGuanosine(iBu)cyanoethylphosphoramidite
10) DMT-dThymidinecyanoethylphosphoramidite
11) Acetonitrile, anhydrous

Tetrazole and acetonitrile are stored over molecular sieves to sequester water.

Phosphoramidites are dissolved in anhydrous acetonitrile (Milligen) at 0.1 g/ml. All other acetonitrile used in the syntheses is "Low-water Acetonitrile" supplied by J. T. Baker Chemical Company (Phillipsburg, NJ). Synthesis columns containing supports charged with an initial base for each of A, C, G, and T are obtained from Milligen in two types, high-loading and low-loading. High-loading columns are used for syntheses of oligo-nts containing up to 60 bases and contain between 35 and 70 micromoles of amidite/g of support. The exact amount varies from lot to lot. Low-loading columns containing between 4 and 7 micromoles amidite/g support are used for syntheses of oligo-nts containing 60 bases or more.

The Milligen 7500 has seven vials from which phosphoramidites may be taken. Normally, the first four contain A, C, T, and G. The other three vials may contain unusual bases such as inosine or mixtures of bases, the so-called "dirty bottle". The standard software allows programmed mixing of two, three, or four bases in equimolar quantities.

When a synthesis is complete, the DNA is removed from the support by incubating the supports in 1 ml of fresh 28-30% ammonium hydroxide solution (EM Science, a division of EM Industries, Inc., Cherry Hill, NJ) for 15 hours at 50 degrees C. The solution is dried under vacuum and the DNA resuspended in 200 microliters of HPLC-grade water (Baker-Analyzed Reagent (R), J.T. Baker Chemical Co.) and is purified by high-pressure liquid chromatography (HPLC) or PAGE.

With low-loading supports, a 65-base-long oligo-nt is typically obtained at 1-2% of theoretical yield, i.e. 10 ug; a 100-base-long oligo-nt is typically obtained in 0.5% of theoretical yield, i.e. 5 ug. With high-loading supports, 1 mg of a 20-base-long oligo-nt is typically obtained.

The present invention is not limited to any particular method of purifying DNA for genetic engineering. HPLC is used for both oligo-nts and fragments of several kb. Alternatively, agarose gel electrophoresis and electroelution on an IBI device (International Biotechnologies, Inc., New Haven, CT) is used to purify large dsDNA fragments. For oligo-nts, PAGE and electroelution with an Epigene device (Epigene Corp., Baltimore, MD) are an alternative to HPLC. One alternative for DNA purification is HPLC on a Waters

(division of Millipore Corporation) HPLC system using the GenPak(TM)-FAX column. A sample of 100 picograms (pg) to 10 ug can be loaded and recovered in 10%-80% yield. The recovery varies with the size and concentration of the DNA, and whether it is single or double stranded. A NAP5 column from Pharmacia (Sweden) is used to desalt DNA eluted from the GenPak column. After passage over the NAP5 column, the DNA solution is vacuum desiccated.

Sec. 6.1: Cloning of Known OSP-ipbd gene into OCV:

In this section, we clone the osp-ipbd gene or the display probe that we have designed. In the preferred method, the synthetic gene is constructed using plasmids that are transformed into bacterial cells by standard methods (MANIS2, p250) or slightly modified standard methods. Alternatively, DNA fragments derived from nature are operably linked to other fragments of DNA derived from nature or to synthetic DNA fragments. In most cases of the preferred method, gene synthesis involves construction of a series of plasmids containing larger and larger segments of the complete gene. Each plasmid that contains a newly added portion of the osp-ipbd gene or of the display probe is tested by restriction digestion. Plasmids having the expected restriction digestion pattern are sequenced in the region of the latest alteration to confirm the synthesis.

If, for convenience, small plasmids were used for gene synthesis, the complete osp-ipbd gene or display probe is subcloned into the OCV at this point.

Sec. 6.2 Cloning of Random DNA (Potential ess) Into Display Probe:

If random DNA and phenotypic selection or screening are used to obtain a GP(IPBD), then we clone random DNA into one of the restriction sites that was designed into the display probe.

The random DNA may be obtained in a variety of ways. Degenerate synthetic DNA is one possibility. Alternatively, pseudorandom DNA may be taken from nature. If, for example, an Sph I site (GCATG/C) has been designed into the display probe at one end of the ipbd fragment, then we would use Nla III (CATG/) to partially digest some DNA that contains a wide variety of sequences, generating a wide variety fragments with CATG 3' overhangs. Preferably, the display probe is designed with different restriction sites at each end of the ipbd gene so that random DNA can be cloned at either end at the user's discretion. The genome of an organism would be a suitable source of DNA with high sequence diversity.

A plasmid carrying the display probe is digested with the appropriate restriction enzyme and the fragmented, random DNA is annealed and ligated by standard methods. The ligated plasmids are used to transform cells that are grown and selected for expression of the antibiotic-resistance gene. Plasmid-bearing GPs are then selected for the display-of-IPBD phenotype by the procedure given in Sec. 15 of the present invention using AfM(IPBD) as if it were the target. Sec. 15 is designed to isolate GP(PBD)s that bind to a target from a large population that do not bind. Use of the procedure of Sec. 15 to isolate a

genetic construction that leads to the display of a single type of IPBD is different from the designed use in one important way: any GP that displays the IPBD will bind tightly and GPs that do not display IPBD will not bind, hence any reasonable amount of AfM(IPBD) on the matrix will identify a successful clone.

As an alternative to selecting GP(IPBD)s through binding to an affinity column, we can isolate colonies or plaques and screen through use of one of the methods listed in Sec. 8 to identify clonal isolates that display IPBD on the GP outer surface.

Sec. 7: Harvest of GPs :

After transforming cells with ligated cloning vectors, we first grow the GPs in non-selective conditions to allow expression of the antibiotic-resistance markers on the cloning vector. After a grow-out, we apply selective pressure to kill untransformed cells.

GPs are harvested by methods appropriate to the GP at hand, generally, centrifugation to pelletize GPs and resuspension of the pellets in sterile medium (cells) or buffer (spores or phage).

Sec. 8: Verification of Display Strategy:

The harvested packages are now tested to determine whether the IPBD is present on the surface. In any tests of GPs for the presence of IPBD on the GP surface, any ions or cofactors known to be essential for the stability of IPBD or AfM(IPBD) must be included at appropriate levels. The tests can be done: a) by

affinity labeling, b) enzymatically, c) spectrophotometrically, d) by affinity separation, or e) by affinity precipitation. The AfM(IPBD) in this step is one picked to have strong affinity (preferably, $K_d < 10^{-11}$ M) for the IPBD molecule and little or no affinity for the wtGP. For example, if BPTI were the IPBD, trypsin, anhydrotrypsin, or antibodies to BPTI could be used as the AfM(BPTI) to test for the presence of BPTI. Anhydrotrypsin, a trypsin derivative with serine 195 converted to dehydroalanine, has no proteolytic activity but retains its affinity for BPTI (AKOH72 and HUBE77).

Preferably, the presence of the IPBD on the surface of the GP is demonstrated through the use of a soluble, labeled derivative of a AfM(IPBD) with high affinity for IPBD. The label could be: a) a radioactive atom such as ^{125}I, b) a chemical entity such as biotin, or 3) a fluorescent entity such as rhodamine or fluorescein. The labeled derivative of AfM(IPBD) is denoted as AfM(IPBD)*. The preferred procedure is:

1) mix AfM(IPBD)* with GPs that are to be tested for the presence of IPBD; conditions of mixing should favor binding of IPBD to AfM(IPBD)*,

2) separate GPs from unbound AfM(IPBD)* by use of:
   a) a molecular sizing filter that will pass AfM(IPBD)* but not GPs,
   b) centrifugation, or
   c) a molecular sizing column (such as Sepharose or Sephadex) that retains free AfM(IPBD)* but not GPs,

3) quantitate the AfM(IPBD)* bound by GPs.

Alternatively, if the IPBD has a known biochemical activity (enzymatic or inhibitory), its presence on the GP can be verified through this activity. For example, if the IPBD were BPTI, then one could use the stoichiometric inactivation of trypsin not only to demonstrate the presence of BPTI, but also to quantitate the amount.

If the IPBD has strong, characteristic absorption bands in the visible or UV that are distinct from absorption by the wtGP, then another alternative for measuring the IPBD displayed on the GP is a spectrophotometric measurement. For example, if IPBD were azurin, the visible absorption could be used to identify GPs that display azurin.

Another alternative is to label the GPs and measure the amount of label retained by immobilized AfM(IPBD). For example, the GPs could be grown with a radioactive precursor, such as $^{32}P_i$ or ^{3}H-thymidine, and the radioactivity retained by immobilized AfM(IPBD) measured.

Another alternative is to use affinity chromatography: the ability of a GP bearing the IPBD to bind a matrix (cf Sec. 15.1) that supports a AfM(IPBD) is measured by reference to the wtGP.

Another alternative for detecting the presence of IPBD on the GP surface is affinity precipitation.

If random DNA has been used, then the procedures of Sec. 15 are used to obtain a clonal isolate that has the display-of-IPBD phenotype. Alternatively, clonal isolates may be screened for the display-of-IPBD phenotype. The tests of this step are applied to one or more of these clonal isolates.

If no isolates that bind to the affinity molecule are obtained we take corrective action as disclosed in Sec. 9.

If one or more of the tests above indicates that the IPBD is displayed on the GP surface, we verify that the binding of molecules having known affinity for IPBD is due to the chimeric osp-ipbd gene through the use of standard genetic and biochemical techniques, such as:

1) transferring the osp-ipbd gene into the parent GP to verify that osp-ipbd confers binding,

2) deleting the osp-ipbd gene from the isolated GP to verify that loss of osp-ipbd causes loss of binding,

3) showing that binding of GPs to AfM(IPBD) correlates with [XINDUCE] (in those cases that expression of osp-ipbd is controlled by [XINDUCE]), and

4) showing that binding of GPs to AfM(IPBD) is specific to the immobilized AfM(IPBD) and not to the support matrix.

Variation of: a) binding of GPs by soluble AfM(IPBD)*, b) absorption caused by IPBD, and c) biochemical reactions of IPBD are linear in the amount of IPBD displayed. Presence of IPBD on the GP surface is indicated by a strong correlation between [XINDUCE] and the reactions that are linear in the amount of IPBD. Leakiness of the promoter is not likely to present problems of high background with assays that are linear in the amount of IPBD. These experiments may be quicker and easier than the genetic tests. Interpreting the effect of [XINDUCE] on binding to a (AfM(IPBD)) column, however, may be problematic unless the regulated promoter is completely repressed in the absence of [XINDUCE]. The affinity retention of GP(IPBD)s is not linear in the number of IPBDs/GP and there may be, for example, little phenotypic difference between GPs bearing 5 IPBDs and GPs bearing 50 IPBDs. The demonstration that binding is to AfM(IPBD) and the genetic tests are essential; the tests with XINDUCE are optional.

We sequence the relevant inbd gene fragment from each of several clonal isolates to determine the construction.

We establish the maximum salt concentration and pH range for which the GP(IPBD) binds the chosen AfM(IPBD). This is preferably done by measuring, as a function of salt concentration and pH, the retention of AfM(IPBD)* on molecular sizing filters that pass AfM(IPBD)* but not GP.

If the IPBD is displayed on the outside of the GP, and if that display is clearly caused by the introduced osp-inbd gene, we proceed to Part II, otherwise we must

analyze the result and adopt appropriate corrective measures.

Sec. 9: Perfecting the Display System:

If we have attempted to fuse an ipbd fragment to a natural osp fragment, our options are :

1) pick a different fusion to the same osp by
   a) using opposite end of osp,
   b) keeping more or fewer residues from osp in the fusion; for example, in increments of 3 or 4 residues,
   c) trying a known or predicted domain boundary,
   d) trying a predicted loop or turn position,

2) pick a different osp, or

3) switch to random DNA method.

If we have just tried the random DNA method unsuccessfully, our options are :

1) choose a different relationship between ipbd fragment and random DNA (ipbd first, random DNA second or vice versa),

2) try a different degree of partial digestion, a different enzyme for partial digestion, a different degree of shearing or a different source of natural DNA, or

3) switch to the natural OSP method.

If all reasonable OSPs of the current GP have been tried and the random DNA method has been tried, both without success, we pick a new GP.

Summary of Part I:

In Part I, we have constructed a GP(IPBD). Although the target material is not picked until Part III, we have already discussed the general properties of targets that influence the choice of IPBD. The user may use the first GP(IPBD) as the starting point for design and construction of other GPs: GP(IPBD1), GP(IPBD2), etc. The different IPBDs might differ in charge and size in such a way that, for any target, at least one of the GP(IPBD)s will be appropriate as a starting point to develop a protein that will bind to that target.

Part II

Sec. 10.0: Affinity Separation Means:

In Part II we optimize an affinity separation system that will be used in Part III to enrich a population of GP(vgPBD)s for those GP(PBD)s that display PBDs with increased affinity for the target.

Affinity chromatography is the preferred means, but FACS, electrophoresis, or other means may also be used.

Sec. 10.1: Optimization of Affinity Chromatography Separation:

For linear gradients, elution volume and eluant concentration are directly related. Changes in eluant concentration cause GPs to elute from the column. Elution volume, however, is more easily measured and specified. It is to be understood that the eluant concentration is the agent causing GP release and that an eluant concentration can be calculated from an elution volume and the specified gradient.

Using a specified elution regime, we compare the elution volumes of GP(IPBD)s with the elution volumes of wtGP on affinity columns supporting AfM(IPBD). Comparisons are made at various: a) amounts of IPBD/GP, b) densities of AfM(IPBD)/(volume of matrix) (DoAMoM), c) initial ionic strengths, d) elution rates, e) amounts of GP/(volume of support), f) pHs, and g) temperatures, because these are the parameters most likely to affect the sensitivity and efficiency of the separation. We then pick those conditions giving the best separation.

We do not optimize pH or temperature; rather we record optimal values for the other parameters for one or more values of pH and temperature. The pH used must be within the range of pH for which GP(IPBD) binds the AfM(IPBD) that is being used in this step. The conditions of intended use, specified by the user (Sec. 11), may include a specification of pH or temperature. If pH is specified, then pH will not be varied in eluting the column (Sec. 15.3). Decreasing pH may, however, be used to liberate bound GPs from the matrix. Similarly, if the intended use specifies a temperature, we will hold the affinity column at the specified temperature during elution, but we might vary the temperature during recovery. If the intended use

specifies the pH or temperature, then we prefer that the affinity separation be optimized for all other parameters at the specified pH and temperature.

In the optimization devised in this step, we preferably use a molecule known to have moderate affinity for the IPBD (K_d in the range 10^{-6} M to 10^{-8} M), for the following reason. When populations of GP(vgPBD)s are fractionated, there will be roughly three subpopulations: a) those with no binding, b) those that have some binding but can be washed off with high salt or low pH, and c) those that bind very tightly and must be rescued in situ. We optimize the parameters to separate (a) from (b) rather than (b) from (c). Let PBD_w be a PBD having weak binding to the target and PBD_s be a PBD having strong binding. Higher DoAMoM might, for example, favor retention of GP(PBD_w) but also make it very difficult to elute viable GP(PBD_s). We will optimize the affinity separation to retain GP(PBD_w) rather than to allow release of GP(PBD_s) because a tightly bound GP(PBD_s) can be rescued by in situ growth. If we find that DoAMoM strongly affects the elution volume, then in part III we may reduce the amount of target on the affinity column when an SBD has been found with moderately strong affinity (K_d on the order of 10^{-7} M) for the target.

In case the promoter of the osp-ipbd gene is not regulated by a chemical inducer, we optimize DoAMoM, the elution rate, and the amount of GP/volume of matrix. If the optimized affinity separation is acceptable, we proceed. If not, we must develop a means to alter the amount of IPBD per GP. Among GPs considered in the present invention, this case could

arise only for spores because regulatable promoters are available for all other systems.

If the amount of IPBD/spore is too high, we could engineer an operator site into the osp-ipbd gene. We choose the operator sequence such that a repressor sensitive to a small diffusible inducer recognizes the operator. Alternatively, we could alter the Shine-Dalgarno sequence to produce a lower homology with consensus Shine-Dalgarno sequences. If the amount of IPBD/spore is too low, we can introduce variability into the promoter or Shine-Dalgarno sequences and screen colonies for higher amounts of IPBD/spore.

In this step, we measure elution volumes of genetically pure GPs that elute from the affinity matrix as sharp bands that can be detected by UV absorption. Alternatively, samples from effluent fractions can be plated on suitable medium (cells or spores) or on sensitive cells (phage) and colonies or plaques counted.

Several values of IPBD/GP, DoAMoM, elution rates, initial ionic strengths, and loadings should be examined. The following is only one of many ways in which the affinity separation could be optimized. We anticipate that optimal values of IPBD/GP and DoAMoM will be correlated and therefore should be optimized together. The effects of initial ionic strength, elution rate, and amount of GP/(matrix volume) are unlikely to be strongly correlated, and so they can be optimized independently.

For each set of parameters to be tested, the column is eluted in a specified manner. For example,

we may use a regime called Elution Regime 1: a KCl gradient runs from 10mM to maximum allowed for the GP(IPBD) viability in 100 fractions of 0.05 V_V, followed by 20 fractions of 0.05 V_V at maximum allowed KCl; pH of the buffer is maintained at the specified value with a convenient buffer such as phosphate, Tris, or MOPS. Other elution regimes can be used; what is important is that the conditions of this optimization be similar to the conditions that are used in Part III for selection for binding to target (Sec. 15.3) and recovery of GPs from the chromatographic system (Sec. 15.4).

When the osp-ipbd gene is regulated by [XINDUCE], IPBD/GP can be controlled by varying [XINDUCE]. Appropriate values of [XINDUCE] depend on the identity of [XINDUCE] and the promoter; if, for example, XINDUCE is isopropylthiogalactoside (IPTG) and the promoter is lacUV5, then [IPTG] = 0, 0.1 uM, 1.0 uM, 10.0 uM, 100.0 uM, and 1.0 mM would be appropriate levels to test. The range of variation of [XINDUCE] is extended until an optimum is found or an acceptable level of expression is obtained.

DoAMoM is varied from the maximum that the matrix material can bind to 1% or 0.1% of this level in appropriate steps. We anticipate that the efficiency of separation will be a smooth function of DoAMoM so that it is appropriate to cover a wide range of values for DoAMoM with a coarse grid and then explore the neighborhood of the approximate optimum with a finer grid.

Several values of initial ionic strength are tested, such as 1.0 mM, 5.0 mM, 10.0 mM and 20.0 mM.

Low ionic strength favors binding between oppositely charged groups, but could also cause GP to precipitate.

The elution rate is varied, by successive factors of 1/2, from the maximum attainable rate to 1/16 of this value. If the lowest elution rate tested gives the best separation, we test lower elution rates until we find an optimum or adequate separation.

The goal of the optimization is to obtain a sharp transition between bound and unbound GPs, triggered by increasing salt or decreasing pH or a combination of both. This optimization need be performed only: a) for each temperature to be used, b) for each pH to be used, and c) when a new GP(IPBD) is created.

Sec. 10.2: Measuring the sensitivity of affinity separation:

Once the values of IPBD/GP, DoAMoM, initial ionic strength, elution rate, and amount of GP/(volume of affinity support) have been optimized, we determine the sensitivity of the affinity separation (C_{sensi}) by the following procedure that measures the minimum quantity of GP(IPBD) that can be detected in the presence of a large excess of wtGP. The user chooses a number of separation cycles, denoted N_{chrom}, that will be performed before an enrichment is abandoned; preferably, N_{chrom} is in the range 6 to 10 and N_{chrom} must be greater than 4. Enrichment can be terminated by isolation of a desired GP(SBD) before N_{chrom} passes.

The measurement of sensitivity is significantly expedited if GP(IPBD) and wtGP carry different selectable markers because such markers allow easy

identification of colonies obtained by plating fractions obtained from the chromatography column. For example, if wtGP carries kanamycin resistance and GP(IPBD) carries ampicillin resistance, we can plate fractions from a column on non-selective media suitable for the GP. Transfer of colonies onto ampicillin- or kanamycin-containing media will determine the identity of each colony.

Mixtures of GP(IPBD) and wtGP are prepared in the ratios of $1:V_{lim}$, where V_{lim} ranges by an appropriate factor (e.g. 1/10) over an appropriate range, typically 10^{11} through 10^{4}. Large values of V_{lim} are tested first; once a positive result is obtained for one value of V_{lim}, no smaller values of V_{lim} need be tested. Each mixture is applied to a column supporting, at the optimal DoAMoM, an AfM(IPBD) having high affinity for IPBD and the column is eluted by the specified elution regime, such as Elution Regime 1. The last fraction that contains viable GPs and an inoculum of the column matrix material are cultured. If GP(IPBD) and wtGP have different selectable markers, then transfer onto selection plates identifies each colony. If GP(IPBD) and wtGP have no selectable markers or the same selectable markers, then a number (e.g. 32) of GP clonal isolates are tested for presence of IPBD by the techniques discussed in Sec. 8. If IPBD is not detected on the surface of any of the isolated GPs, then GPs are pooled from: a) the last few (e.g. 3 to 5) fractions that contain viable GPs, and b) an inoculum taken from the column matrix. The pooled GPs are cultured and passed over the same column and enriched for GP(IPBD) in the manner described. This process is repeated until N_{chrom} passes have been performed, or until the IPBD has been detected on the GPs. If

GP(IPBD) is not detected after N_{chrom} passes, V_{lim} is decreased and the process is repeated.

Once a value for V_{lim} is found that allows recovery of GP(IPBD)s, the factor by which V_{lim} is varied is reduced and additional values are tested until V_{lim} is known to within a factor of two.

C_{sensi} equals the highest value of V_{lim} for which the user can recover GP(IPBD) within N_{chrom} passes. The number of chromatographic cycles (K_{cyc}) that were needed to isolate GP(IPBD) gives a rough estimate of C_{eff}; C_{eff} is approximately the K_{cyc}th root of Vlim:

$$C_{eff} = \text{(approx.) } \exp(\log_e(V_{lim})/K_{cyc})$$

For example, if V_{lim} were 4.0×10^8 and three separation cycles were needed to isolate GP(IPBD), then C_{eff} = (approx.) 736.

Sec. 10.3: Measuring the efficiency of separation :

To determine C_{eff} more accurately, we determine the ratio of GP(IPBD)/wtGP loaded onto an AfM(IPBD) column that yields approximately equal amounts of GP(IPBD) and wtGP after elution. We prepare mixtures of GP(IPBD) and wtGP in ratios GP(IPBD):wtGP :: 1:Q; we start Q at twenty times the approximate C_{eff} found in Sec. 10.2. A 1:Q mixture of GP(IPBD) and wtGP is applied to a AfM(IPBD) column and eluted by the specified elution regime, such as Elution Regime 1. A sample of the last fraction that contains viable GPs is plated at a dilution that gives well separated colonies or plaques. The presence of IPBD or the osp-ipbd gene in each colony or plaque can be determined by a number

of standard methods, including: a) use of different selectable markers, b) nitrocellulose filter lift of GPs and detection with Afm(IPBD)* (AUSU87), or c) nitrocellulose filter lift of GPs and detection with radiolabeled DNA that is complementary to the osp-ipbd gene (AUSU87). Let F be the fraction of GP(IPBD) colonies found in the last fraction containing viable GPs. When a Q is found such that .20 < F < .80, then

$$C_{eff} = Q * F.$$

If F < 0.2, then we reduce Q by an appropriate factor (e.g. 1/10) and repeat the procedure. If F > 0.8, then we increase Q by an appropriate factor (e.g. 2) and repeat the procedure.

Sec. 10.4: Other Separation Means

Other separation means are optimized in a manner parallel to the used for affinity chromatography.

FACS is likely to be most appropriate for bacterial cells and spores because the sensitivity of the machines requires approximately 1000 molecules of fluorescent label bound to each GP to accomplish a separation. An appropriate commercial FACS machine is a FACStar from Beckton-Dickinson, Mountain View, CA. To optimize FACS separation of GPs, we use a derivative of Afm(IPBD)A that is labeled with a fluorescent molecule, denoted Afm (IPBD)*. The variables that must be optimized include: a) amount of IPBD/GP, b) concentration of Afm(IPBD)*, c) ionic strength, d) concentration of GPs, and e) parameters pertaining to operation of the FACS machine. Because Afm(IPBD)* and GPs interact in solution, the binding will be linear in

both [Afm(IPBD)*] and [displayed IPBD]. Preferably, these two parameters are varied together. The other parameters can be optimized independently. The sensitivity and efficiency of the FACS separation are determined in a manner parallel to those used for chromatography.

Electrophoresis is most appropriate to bacteriophage because of their small size. Serwer (SERW87) has reviewed use of agarose-gel electrophoresis to separate phage based on charge. Electrophoresis is a preferred separation means if the target is so small that chemically attaching it to a column or to a fluorescent label would essentially change the entire target. For example, chloroacetate ions contain only seven atoms and would be essentially altered by any linkage. GPs that bind chloroacetate would become more negatively charged than GPs that do not bind the ion and so these classes of GPs could be separated.

The parameters to optimize for electrophoresis include: a) IPBD/GP, b) concentration of gel material, e.g. agarose, c) concentration of Afm (IPBD), d) ionic strength, e) size, shape, and cooling capacity of the electrophoresis apparatus, f) voltages and currents, and f) concentration of GPs. Preferably, IPBD/GP and [Afm(IPBD)] are varied at the same time and other parameters are optimized independently.

In Part II we have determined optimal conditions for separating GPs based on proteins displayed on the GP surface. We have also determined the capabilities of the affinity separation system. Knowledge of these

capabilities allows us to choose appropriate levels of variegation in Part III.

Part III

Sec. 11.0: Choice of target material :

Any material may be chosen as target material, subject only to the following restrictions:

If affinity chromatography is to be used, then:

1) the molecules of the target material must be of sufficient size and chemical reactivity to be applied to a solid support suitable for affinity separation,

2) after application to a matrix, the target material must not react with water,

3) after application to a matrix, the target material must not bind or degrade proteins in a non-specific way, and

4) the molecules of the target material must be sufficiently large that attaching the material to a matrix allows enough unaltered surface area (generally at least 500 $Å^2$, excluding the atom that is connected to the linker) for protein binding.

If FACS is to be used as the affinity separation means, then:

1) the molecules of the target material must be of sufficient size and chemical reactivity to be conjugated to a suitable fluorescent dye or the target must itself be fluorescent,

2) after any necessary fluorescent labeling, the target must not react with water,

3) after any necessary fluorescent labeling, the target material must not bind or degrade proteins in a non-specific way, and

4) the molecules of the target material must be sufficiently large that attaching the material to a suitable dye allows enough unaltered surface area (generally at least 500 A^2, excluding the atom that is connected to the linker) for protein binding.

If affinity electrophoresis is to be used, then:

1) the target must either be charged or of such a nature that its binding to a protein will change the charge of the protein,

2) the target material must not react with water,

3) the target material must not bind or degrade proteins in a non-specific way, and

4) the target must be compatible with a suitable gel material.

Possible target materials include, but are not limited to:

1) horse heart myoglobin
2) cholesterol
3) O-antigen of Salmonella enteritidis
4) yeast phenylalanyl tRNA
5) asbestos
6) alpha-fetoprotein
7) ras proteins
8) low density lipoprotein
9) prostaglandin PGE2
10) alpha interferon
11) melittin
12) Bordetella pertussis adenylate cyclase toxin
13) aflatoxin B_1
14) aspartame
15) haem
16) bilirubin
17) morphine
18) codeine
19) dichlorodiphenyltrichlorethane (DDT)
20) benzo(a)pyrene
21) actinomycin D
22) any retroviral pol protease
23) any retroviral gag protease
24) B. pertussis agglutinogen
25) B. pertussis dermonecrotic toxin
26) N. gonorrhoeae pilus protein
27) fibril or flagellar protein from any of several spirochaete bacterial species, e.g. organisms causing syphilis, Lyme disease, or relapsing fever
28) E. coli enterotoxin protein
30) Pseudomonas aeruginosa hemolysin
31) zeolites
32) cellulose

33) hydroxylapatite
34) DNA of a defined sequence
35) fibrin
36) tumor necrosis factor
37) specific monoclonal antibodies

A supply of several milligrams of pure target material is desired. Impure target material could be used, but one might obtain a protein that binds to a contaminant instead of to the target.

The following information about the target material is highly desirable:

1) stability as a function of temperature, pH, and ionic strength,

2) stability with respect to chaotropes such as urea or guanidinium Cl,

3) pI,

4) molecular weight,

5) requirements for prosthetic groups or ions, such as haem or Ca^{+2}, and

6) proteolytic activity, if any.

In addition to this most desirable information, it is useful to know: 1) the target's sequence, if the target is a macromolecule, 2) the 3D structure of the target, 3) enzymatic activity, if any, and 4) toxicity, if any.

The user of the present invention specifies certain parameters of the intended use of the binding protein:

1) the acceptable temperature range,

2) the acceptable pH range,

3) the acceptable concentrations of ions and neutral solutes,

4) the maximum acceptable dissociation constant for the target and the SBD:

$$K_T = [\text{Target}][\text{SBD}]/[\text{Target:SBD}]$$

In some cases, the user may require discrimination between T, the target, and N, some non-target. Let

$K_T = [T][SBD]/[T:SBD]$, and
$K_N = [N][SBD]/[N:SBD]$,

then $K_T/K_N = ([T][N:SBD])/([N][T:SBD])$.

The user then specifies a maximum acceptable value for the ratio K_T/K_N.

The target material must be stable under the specified conditions of pH, temperature, and solution conditions.

If the target material is a protease, one must consider the following points:

1) a highly specific protease can be treated like any other target,

2) a general protease, such as subtilisin, may degrade the OSPs of the GP including OSP-PBDs; there are several alternative ways of dealing with general proteases, including: a) a chemical inhibitor may be used to prevent proteolysis (e.g. phenylmethylfluorosulfate (PMFS) that inhibits serine proteases), b) one or more active-site residues may be mutated to create an inactive protein (e.g. a serine protease in which the active serine is mutated to alanine), or c) one or more active-site amino-acids of the protein may be chemically modified to destroy the catalytic activity (e.g. a serine protease in which the active serine is converted to anhydroserine),

3) SBDs selected for binding to a protease need not be inhibitors: SBDs that happen to inhibit the protease target are a fairly small subset of SBDs that bind to the protease target,

4) the more we modify the target protease, the less like we are to obtain an SBD that inhibits the target protease, and

5) if the user requires that the SBD inhibit the target protease, then the active site of the target protease must not be modified any more than necessary; inactivation by mutation or chemical modification are preferred methods of inactivation and a protein protease inhibitor becomes a prime candidate for IPBD. For example, BPTI could be mutated, by the methods of the present invention,

to bind to proteases other than trypsin (TANK77 and TSCH87).

Sec. 12.0: Choice of GP(IPBD) :

The user must pick a GP(IPBD) that is suitable to the chosen target according to the criteria of Sec. 2. It is anticipated that a small collection of a GP(IPBD)s can be assembled such that, for any chosen target, at least one member of the collection will be a suitable starting point for engineering a protein that binds to the chosen target by the methods of the present invention.

If the pH, temperature, or other parameters of the intended use of the selected SBD differ markedly from the conditions used to optimize the affinity separation for the chosen GP(IPBD), then the user should optimize the affinity separation for conditions appropriate to the intended use by the methods described in Part II.

Sec. 13.0: Identification of Family of PBDs, Related to PPBD, to Be Generated

Sec. 13.1: Choosing residues on IPBD (or other PPBD) to vary:

We choose residues in the IPBD to vary through consideration of several factors, including: a) the 3D structure of the IPBD, b) sequences homologous to IPBD, and c) modeling of the IPBD and mutants of the IPBD. Because the number of residues that could strongly influence binding is always greater than the number that can be varied simultaneously, the user must pick a subset of those residues to vary at one time. The user

must also pick trial levels of variegation and calculate the abundances of various sequences. The list of varied residues and the level of variegation at each varied residue are adjusted until the composite variegation is commensurate with C_{sensi} and M_{ntv}.

We now consider the principles that guide our choice of residues of the IPBD to vary. A key concept is that only structured proteins exhibit specific binding, i.e. can bind to a particular chemical entity to the exclusion of most others. Thus the residues to be varied are chosen with an eye to preserving the underlying IPBD structure. Substitutions that prevent the PBD from folding will cause GPs carrying those genes to bind indiscriminately so that they can easily be removed from the population.

Burial of hydrophobic surfaces so that bulk water is excluded is one of the strongest forces driving the binding of proteins to other molecules. Bulk water can be excluded from the region between two molecules only if the surfaces are complementary. We must test as many surfaces as possible to find one that is complementary to the target. The selection-through-binding isolates those proteins that are more nearly complementary to some surface on the target. The effective diversity of a variegated population is measured by the number of different surfaces, rather than the number of protein sequences. Thus we should maximize the number of surfaces generated in our population, rather than the number of protein sequences.

In hypothetical example 1, we consider a hypothetical PBD, shown in Figure 4 binding to a

hypothetical target. Figure 4 is a 2D schematic of 3D objects; by hypothesis, residues 1, 2, 4, 6, 7, 13, 14, 15, 20, 21, 22, 27, 29, 31, 33, 34, 36, 37, 38, and 39 of the IPBD are on the 3D surface of the IPBD, even though shown well inside the circle. Proteins do not have distinct, countable faces. Therefore we define an "interaction set" to be a set of residues such that all members of the set can simultaneously touch one molecule of the target material without any atom of the target coming closer than van der Waals distance to any main-chain atom of the IPBD. The concept of a residue "touching" a molecule of the target is discussed below. One hypothetical interaction set, Set A, in Figure 4 comprises residues 6, 7, 20, 21, 22, 33, and 34, represented by squares. Another hypothetical interaction set, Set B, comprises residues 1, 2, 4, 6, 31, 37, and 39, represented by circles.

If we vary one residue, number 21 for example, through all twenty amino acids, we obtain 20 protein sequences and 20 different surfaces for interaction set A. Note that residue 6 is in two interaction sets and variation of residue 6 through all 20 amino acids yields 20 versions of interaction set A and 20 versions of interaction set B.

Now consider varying two residues, each through all twenty amino acids, generating 400 protein sequences. If the two residues varied were, for example, number 1 and number 21, then there would be only 40 different surfaces because interaction set A does not depend on residue 1 and interaction set B does not depend on residue 21. If the two residues varied, however, were number 7 and number 21, then 400 surfaces would be generated.

If N spatially separated residues are varied at one time, 20 x N surfaces are generated. Variation of N residues in the same interaction set yields 20^N surfaces. For example, if N = 7, variation of separated residues yields 140 surfaces while variation of interacting residues yields $20^7 = 6.4 \times 10^9$ surfaces. Thus, to maximize the number of surfaces generated when N residues are varied, all residues should be in the same interaction set because variation of several residues in one interaction set generates an exponential number of surfaces while variation of spatially separated surface residues generates only a linear number.

The amount of surface area buried in strong protein-protein interactions ranges from 1000 A^2 to 2000 A^2, as summarized by Schulz and Schirmer (SCHU79, p103ff). Individual amino acids have total surface areas that depend mostly on type of amino acid and weakly on conformation. These areas range from about 180 A^2 for glycine to about 360 A^2 for tryptophan. Averages of total surface area by amino acid type and maximum exposed surface area of each amino acid type for two typical proteins, hen egg white lysozyme (HEWL) and T4 lysozyme (T4L), are shown in Table 6. From these exposures, one can calculate that 1000 A^2 on a protein surface comprises between 4 and 30 amino acids, depending on the amino acid types and the protein 3D structure. Varied amino acid sequences, as found in actual proteins, involve between 10 and 25 residues in forming 1000 A^2 of protein surface. Schulz and Schirmer estimate that 100 A^2 of protein surface can exhibit as many as 1000 different specific patterns (SCHU79, p105). The number of surface patterns rises

exponentially with the area that can be varied independently. One of the BPTI structures recorded in the Brookhaven Protein Data Bank (6PTI), for example, has a total exposed surface area of 3997 A^2 (using the method of Lee and Richards (LEEB71) and a solvent radius of 1.4 A and atomic radii as shown in Table 7). If we could vary this surface freely and if 100 A^2 can produce 1000 patterns, we could construct 10^{120} different patterns by varying the surface of BPTI! This calculation is intended only to suggest the huge number of possible surface patterns based on a common protein backbone.

One protein framework cannot, however, display all possible patterns over any one particular 100 A^2 of surface merely by replacement of the side groups of surface residues. The protein backbone holds the varied side groups in approximately constant locations so that the variations are not independent. We can, nevertheless, generate a vast collection of different protein surfaces by varying those protein residues that face the outside of the protein.

Figure 5 shows BPTI in contact with myoglobin. From this we can see that residues 3, 7, 8, 10, 13, 39, 41, and 42 can all simultaneously contact a molecule the size and shape of myoglobin. Figure 5 also shows that residue 49 can not touch a single myoglobin molecule simultaneously with any of the first set even though all are on the surface of BPTI. It is not the intent of the present invention, however, to use models to determine which part of the target molecule will actually be the site of binding by PBD.

If cassette mutagenesis is picked, the protein residues to be varied are, preferably, close enough together in sequence that the variegated DNA (vgDNA) encoding all of them can be made in one piece. The present invention is not limited to a particular length of vgDNA that can be synthesized. With current technology, a stretch of 60 amino acids (180 DNA bases) can be spanned.

Further, when there is reason to mutate residues further than sixty residues apart, one can use other mutational means, such as single-stranded-oligonucleotide-directed mutagenesis (BOTS85) using two or more mutating primers.

Alternatively, to vary residues separated by more than sixty residues, two cassettes may be mutated as follows:

1) vg DNA having a low level of variegation (for example, 20 to 400 fold variegation) is introduced into one cassette in the OCV,

2) cells are transformed and cultured,

3) vg OCV DNA is obtained,

4) a second segment of vgDNA is inserted into a second cassette in the OCV, and

5) cells are transformed and cultured, GPs are harvested and subjected to selection-through-binding.

The composite level of variation must not exceed the prevailing capabilities to a) produce very large numbers of independently transformed cells or b) detect small components in a highly varied population. The limits on the level of variegation are discussed in Sec. 13.2.

Here we assemble the data about the IPBD and the target that are useful in deciding which residues to vary in the variegation cycle:

1) 3D structure, or at least a list of residues on the surface of the IPBD,

2) list of sequences homologous to IPBD, and

3) model of the target molecule or a stand-in for the target.

These data and an understanding of the behavior of different amino acids in proteins will be used to answer two questions:

1) which residues of the IPBD are on the outside and close enough together in space to touch the target simultaneously?

2) which residues of the IPBD can be varied with high probability of retaining the underlying IPBD structure?

Although an atomic model of the target material (obtained through X-ray crystallography, NMR, or other means) is preferred in such examination, it is not

necessary. For example, if the target were a protein of unknown 3D structure, it would be sufficient to know the molecular weight of the protein and whether it were a soluble globular protein, a fibrous protein, or a membrane protein. Physical measurements, such as low-angle neutron diffraction, can determine the overall molecular shape, viz. the ratios of the principal moments of inertia. One can then choose a protein of known structure of the same class and similar size and shape to use as a molecular stand-in and yardstick. It is not essential to measure the moments of inertia of the target because at low resolution, all proteins of a given size and class look much the same. The specific volumes are the same, all are more or less spherical and therefore all proteins of the same size and class have about the same radius of curvature. The radii of curvature of the two molecules determine how much of the two molecules can come into contact.

Several graphical and computational tools that are needed or useful. The most appropriate method of picking the residues of the protein chain at which the amino acids should be varied is by viewing, with interactive computer graphics, a model of the IPSD. A stick-figure representation of molecules is preferred. A suitable set of hardware is an Evans & Sutherland PS390 graphics terminal (Evans & Sutherland Corporation, Salt Lake City, UT) and a MicroVAX II supermicro computer (Digital Equipment Corp., Maynard, MA). The computer should, preferably, have at least 150 megabytes of disk storage, so that the Brookhaven Protein Data Bank can be kept on line. A FORTRAN compiler, or some equally good higher-level language processor is preferred for program development. Suitable programs for viewing and manipulating protein

models include: a) PS-FRODO, written by T. A. Jones (JONE85) and distributed by the Biochemistry Department of Rice University, Houston, TX; and b) PROTEUS, developed by Dayringer, Tramantano, and Fletterick (DAYR86). Important features of PS-FRODO and PROTEUS that are needed to view and manipulate protein models for the purposes of the present invention are the abilities to: 1) display molecular stick figures of proteins and other molecules, 2) zoom and clip images in real time, 3) prepare various abstract representations of the molecules, such as a line joining C_{alpha}s and side group atoms, 4) compute and display solvent-accessible surfaces reasonably quickly, 5) point to and identify atoms, and 6) measure distance between atoms.

In addition, one could use theoretical calculations, such as dynamic simulations of proteins, to estimate whether a substitution at a particular residue of a particular amino-acid type might produce a protein of approximately the same 3D structure as the parent protein. Such calculations might also indicate whether a particular substitution will greatly affect the flexibility of the protein; calculations of this sort may be useful but are not required.

Sec. 13.1.1: The principal set:

In this section we pick a principal set of residues of the IPBD to vary. Using the knowledge of which residues are on the surface of the IPBD (as noted above), we pick residues that are close enough together on the surface of the IPBD to touch a molecule of the target simultaneously without having any IPBD main-chain atom come closer than van der Waals distance

(viz. 4.0 to 5.0 Å) from any target atom. For the purposes of the present invention, a residue of the IPBD "touches" the target if: a) a main-chain atom is within van der Waals distance, viz. 4.0 to 5.0 Å of any atom of the target molecule, or b) the C_{beta} is within D_{cutoff} of any atom of the target molecule so that a side-group atom could make contact with that atom. Because side groups differ in size (cf. Table 35), some judgment is required in picking D_{cutoff}. In the preferred embodiment, we will use D_{cutoff} = 8.0 Å, but other values in the range 6.0 Å to 10.0 Å could be used. If IPBD has G at a residue, we construct a pseudo C_{beta} with the correct bond distance and angles and judge the ability of the residue to touch the target from this pseudo C_{beta}.

Alternatively, we choose a set of residues on the surface of the IPBD such that the curvature of the surface defined by the residues in the set is not so great that it would prevent contact between all residues in the set and a molecule of the target. This method is appropriate if the target is a macromolecule, such as a protein, because the PBDs derived from the IPBD will contact only a part of the macromolecular surface. The surfaces of macromolecules are irregular with varying curvatures. If we pick residues that define a surface that is not too convex, then there will be a region on a macromolecular target with a compatible curvature.

In addition to the geometrical criteria, we prefer that there be some indication that the underlying IPBD structure will tolerate substitutions at each residue in the principal set of residues. Indications could come from various sources, including: a) homologous

sequences, b) static computer modeling, or c) dynamic computer simulations.

The residues in the principal set need not be contiguous in the protein sequence. The exposed surfaces of the residues to be varied do not need to be connected. We require only that the amino acids in the residues to be varied all be capable of touching a molecule of the target material simultaneously without having atoms overlap. If the target were, for example, horse heart myoglobin, and if the IPBD were BPTI, any set of residues in one interaction set of BPTI defined in Table 34 could be picked.

Preferably, the principal set contains eight to sixteen residues. This number of residues allows sufficient variability that a surface that is complementary to the target can be found, but is small enough that a significant fraction of the surface can be varied at one time.

Sec. 13.1.2: The secondary set:

The secondary set comprises those residues not in the primary set that touch residues in the primary set. These residues might be excluded from the primary set because: a) the residue is internal, b) the residue is highly conserved, or c) the residue is on the surface, but the curvature of the IPBD surface prevents the residue from being in contact with the target at the same time as one or more residues in the primary set.

Internal residues are frequently conserved and the amino acid type can not be changed to a significantly different type without substantial risk that the

protein structure will be disrupted. Nevertheless, some conservative changes of internal residues, such as I to L or F to Y, are tolerated. Such conservative changes affect the detail placement and dynamics of adjacent protein residues and such variation may be useful once an SBD is found.

Surface residues in the secondary set are most often located on the periphery of the principal set. Such peripheral residues can not make direct contact with the target simultaneously with all the other residues of the principal set. The charge on the amino acid in one of these residues could, however, have a strong effect on binding. Once an SBD is found, it is appropriate to vary the charge of some or all of these residues. For example, the variegated codon containing equimolar A and G at base 1, equimolar C and A at base 2, and A at base 3 yields amino acids T, A, K, and E with equal probability.

Sec. 13.1.3: Choice of residues to vary initially:

Choice of residues in the primary and secondary set is based on: a) geometry of the IPBD and the geometrical relationship between the IPBD and the target (or a stand-in for the target) in a hypothetical complex, and b) sequences of proteins homologous to the IPBD. In this section we pick a subset of the residues in the primary and secondary sets, based on geometry and on the maximum allowed level of variegation that assures progressivity. The allowed level of variegation determines how many residues can be varied at once; geometry determines which ones.

The user may pick residues to vary in many ways; the following is a preferred manner. Pairs of residues are picked that are diametrically opposed across the face of the principal set. Two such pairs are used to delimit the surface, up/down and right/left. Alternatively, three residues that form an inscribed triangle, having as large an area as possible, on the surface are picked. One to three other residues are picked in a checkerboard fashion across the interaction surface. Choice of widely spaced residues to vary creates the possibility for high specificity because all the intervening residues must have acceptable complementarity before favorable interactions can occur at widely-separated residues.

The number of residues picked is coupled to the range through which each can be varied by the restrictions discussed in Sec. 13.2. In the first round, we do not assume any binding between IPBD and the target and so progressivity is not an issue. At the first round, the user may elect to produce a level of variegation such that each molecule of vgDNA is potentially different through, for example, unlimited variegation of 10 codons (20^{10} approx. = 10^{13}). One run of the DNA synthesizer produces approximately 10^{13} molecules of length 100 nts. Inefficiencies in ligation and transformation will reduce the number of proteins actually tested to between 10^7 and 5×10^6. Multiple replications of the process with such very high levels of variegation will not yield repeatable results; the user must decide whether this is important.

Sec. 13.2: Range of variation at Each Site of Mutation:

Having picked which residues to vary, we must now decide the range of amino acids to allow at each variable residue. The total level of variegation is the product of the number of variants at each varied residue. Each varied residue can have a different scheme of variegation, producing 2 to 20 different possibilities. We require that the process be progressive, i.e. each variegation cycle produces a better starting point for the next variegation cycle than the previous cycle produced.

N.B.: Setting the level of variegation such that the pobd and many sequences related to the pobd sequence are present in detectable amounts insures that the process is progressive. If the level of variegation is so high that the pobd sequence is present at such low levels that there is an appreciable chance that no transformant will display the PPBD, then the best SBD of the next round could be worse than the PPBD. At excessively high level of variegation, each round of mutagenesis is independent of previous rounds and there is no assurance of progressivity. This approach can lead to valuable binding proteins, but repetition of experiments with this level of variegation will not yield progressive results. Excessive variation is not preferred.

Hypothetical example 2 considers the effects of the level of variegation on the progressivity of the process of the present invention. Figure 6 is a schematic view of a hypothetical eight-residue binding

surface of a PBD comprising residues 11, 24, 25, 30, 34, 42, 44, and 47 of a hypothetical protein. Each polygon represents the exposed portion of one residue. By hypothesis, there exists at least one protein, shown in Figure 6e, having a specific amino acid in each of the eight residues that will bind to the target, but we do not, at first, know what that sequence is.

The IPBD, shown in Figure 6a, may have none of the optimal amino acids on its surface. Because we begin with no information, our initial estimate is that all amino acids have equal likelihood of being the best at each of the eight residues.

By hypothesis, the genetic engineering system of hypothetical example 2 has $M_{ntv} = 10^7$ and the selection-through-binding system has $C_{sensi} = 10^7$. Also by hypothesis, the variegation method can produce all amino acids at a given residue with equal probability.

In the first variegation, we vary residues 11, 24, 25, 34, and 44 through all twenty amino acids, producing $20^5 = 3.2 \times 10^6$ sequences. The capabilities of the genetic engineering system allows all these sequences to be present in the selection step and the selection system can detect 1 GP in 10^7. By hypothesis, we isolate a GP carrying an sbd gene that encodes the first SBD, shown in Figure 6b, that has improved binding for the target and has the amino acid sequence W11-F24-E25-G30-D34-E42-P44-T47. This amino acid sequence becomes the parental sequence to the next variegation. After the first variegation and selection, the evidence favors W11, F24, E25, D34, and P44 as optimal amino acids at their respective

residues. That residues 30, 42, and 47 were not varied has two implications:

1) we still have no information about which amino acid is optimal at these residues, and

2) the amino acids selected at the varied residues are optimal, given the identities of the amino acids in the non-varied residues; when residues 30, 42, and 47 are varied, our estimate of the optimal amino acids in other residues may change.

Now consider two versions of a variegation that take the first intermediate SBD as parent and that might get us closer to the optimal SBD.

In the first version of the second variegation, we vary only five residues, producing 3.2×10^6 sequences, all of which are expressed and subjected to selection-through-binding. We vary residues 30, 42, and 47 because they were not varied previously. We also vary two other residues so that as many surfaces as possible are tested: residues 24 and 44 are chosen. Suppose that we isolate a GP that carries an sbd gene encoding the amino acid sequence W11-L24-E25-I30-D34-R42-P44-K47, shown in Figure 6c. Consider the reason that D is retained at residue 34. We know that all the sequences W11-L24-E25-I30-x34-R42-P44-K47 (where x runs through all twenty amino acids) were tested and therefore can conclude with improved confidence that D34 is optimal, given the rest of the selected sequence. Now consider the change at residue 24 from F to L. We know that all the sequences W11-x24-E25-I30-D34-R42-P44-K47 were tested and we can conclude that L24 is optimal, given the rest of the sequence. At each of the varied

residues, we gain information about which amino acids are optimal at each varied residue under the conditions imposed.

In the second version, we will vary residues 11, 24, 30, 34, 42, and 47, each through all twenty amino acids, producing $20^6 = 6.4 \times 10^7$ possible different sequences. Our hypothesis is that only 1.0×10^7 of these sequences are produced and subjected to selection. Because only 15.6% of the programmed sequences are actually subjected to selection, it is likely that the parental sequence, W11-F24-E25-G30-D34-E42-P44-T47, is not present in the selection step and there is, consequently, no assurance that the best SBD binds more tightly to target than did the parental PBD. Suppose that we isolate a GP that carries an sbd gene encoding the amino acid sequence V11-R24-E25-Q30-D34-R42-P44-D47, shown in Figure 6d. Consider the reason that D is retained at residue 34. Is it that D is optimal, or is it that, by chance, the sequence encoding the optimal amino acid, x, was not present as V11-R24-E25-Q30-x34-R42-P44-D47 in the sample? We do not know and therefore can not conclude that D34 is optimal. Furthermore, retaining an amino acid can not move us toward the optimal sequence. Now consider the change at residue 24 from F to R. Was V11-R24-E25-Q30-D34-R42-P44-D47 selected because R24 is optimal in the presence of V11- -E25-Q30-D34-R42-P44-D47, or was V11-R24-E25-Q30-D34-R42-P44-D47 selected because V11-F24-E25-Q30-D34-R42-P44-D47 was not present to be selected? Again, we do not know and can not conclude that R24 is an improvement, i.e. we can not conclude that R24 is more likely to be optimal than is F24. In both cases, we lose information about which amino acids belong at each residue. We may have obtained an SBD with

superior binding to the target. Another variegation cycle at this level of variegation, however, may produce a better protein or a worse protein and the process is not progressive.

Let us contrast versions 1 and 2 of the second variegation. In version 1, we retained more information, viz. that W11 allows improved binding, and therefore our selection of K47 incorporates the information obtained in the previous rounds. In version 2 of the second variegation, we discarded the information that W11 allows stronger binding than Y11.

Progressivity is not an all-or-nothing property. So long as most of the information obtained from previous variegation cycles is retained and many different surfaces that are related to the PPBD surface are produced, the process is progressive. If the level of variegation is so high that the ppbd gene may not be detected, the assurance of progressivity diminishes. If the probability of recovering PPBD is negligible, then the probability of progressive behavior is also negligible.

An opposing force in our design considerations is that PBDs are useful in the population only up to the amount that can be detected; any excess above the detectable amount is wasted. Thus we produce as many surfaces related to PPBD as possible within the constraint that the PPBD be detectable.

We defer specification of exactly how much variegation is allowed until we have: a) specified real nt distributions for a variegated codon, and b)

examined the effects of discrepancies between specified nt distributions and actual nt distributions.

Sec. 13.3: Design of vgDNA Encoding PBD Family:

We must now decide how to distribute the variegation within the codons for the residues to be varied. These decisions are influenced by the nature of the genetic code. When vgDNA is synthesized, variation at the first base of a codon creates a population containing amino acids from the same column of the genetic code table (as shown in the Table 3-6 on p87 of WATS87); variation at the second base of the codon creates a population containing amino acids from the same row of the genetic code table; variation at the third base of the codon creates a population containing amino acids from the same box. If two or three bases in the same codon are varied, the pattern is more complicated. Work with 3D protein structural models may suggest definite sets of amino acids to substitute at a given residue, but the method of variation may require either more or fewer kinds of amino acids be included. For example, examination of a model might suggest substitution of N or Q at a given residue. Combinatorial variation of codons requires that mixing N and Q at one location also include K and H as possibilities at the same residue. One must choose to put: 1) N only, 2) Q only, or 3) a mixture of N, K, H, and Q. The present invention does not rely on accurate predictions of which amino acids should be placed at each residue, rather attention is focused on which residues should be varied.

There are many ways to generate diversity in a protein. (See RICH86, CARU85, and CLIP86.) One extreme

case is that one or a few residues of the protein are varied as much as possible (inter alia see CARU85, CARU87, RICH36, and WHAR86). We will call this limit "Focused Mutagenesis". Focused Mutagenesis is appropriate when the IPBD or other PPBD shows little or no binding to the target, as at the beginning of the search for a protein to bind to a new target material. When there is no binding between the PPBD and the target, we preferably pick a set of five to seven residues on the surface and vary each through all 20 possibilities.

An alternative plan of mutagenesis ("Diffuse Mutagenesis") that may be useful is to vary many more residues through a more limited set of choices (See Vershon et al., Ch15 of INOU86 and PAKU86). This can be accomplished by spiking each of the pure nts activated for DNA synthesis (e.g. nt-phosphoramidites) with a small amount of one or more of the other activated nts. Contrary to general practice, the present invention sets the level of spiking so that only a small percentage (1% to .00001%, for example) of the final product will contain the initial DNA sequence. This will insure that many single, double, triple, and higher mutations occur, but that recovery of the basic sequence will be a possible outcome. Let N_b be the number of bases to be varied, and let Q be the fraction of all sequences that should have the parental sequence, then M, the fraction of the mixture that is the majority component, is

$$M = \exp(\log_e(Q)/N_b) = 10^{(\log_{10}(Q)/N_b)}.$$

If, for example, thirty base pairs on the DNA chain were to be varied and 1% of the product is to

have the parental sequence, then each mixed nt substrate should contain 86% of the parental nt and 14% of other nts. Table 8 shows the fraction (fn) of DNA molecules having n non-parental bases when 30 bases are synthesized with reagents that contain fraction M of the majority component. When M=.61096, f24 and higher are less than 10^{-8}. The entry "most" in Table 8 is the number of changes that has the highest probability. Note that substantial probability for multiple substitutions only occurs if the fraction of parental sequence (f0) is allowed to drop to around 10^{-6}. Mutagenesis of this sort can be applied to any part of the protein at any time, but is most appropriate when some binding to the target has been established. The N_b base pairs of the DNA chain that are synthesized with mixed reagents need not be contiguous. They are picked so that between $N_b/3$ and N_b codons are affected to various degrees. The residues picked for mutation are picked with reference to the 3D structure of the IFBD, if known. For example, one might pick all or most of the residues in the principal and secondary set. We may impose restrictions on the extent of variation at each of these residues based on homologous sequences or other data. The mixture of non-parental nts need not be random, rather mixtures can be biased to give particular amino acid types specific probabilities of appearance at each codon. For example, one residue may contain a hydrophobic amino acid in all known homologous sequences; in such a case, the first and third base of that codon would be varied, but the second would be set to T. Other examples of how this might be done will be given in the Detailed Example. This diffuse structure-directed mutagenesis will reveal the subtle changes possible in protein backbone associated with conservative interior changes,

such as V to I, as well as some not so subtle changes that require concomitant changes at two or more residues of the protein.

For Focused Mutagenesis, we now consider the distribution of nts that will be inserted at each variegated codon. Each codon could be programmed differently. If we have no information indicating that a particular amino acid or class of amino acid is appropriate, we strive to substitute all amino acids with equal probability because representation of one pbd above the detectable level is wasteful. Equal amounts of all four nts at each position in a codon yields the amino acid distribution:

4/64 A	2/64 C	2/64 D	2/64 E	2/64 F	4/64 G
2/64 H	3/64 I	2/64 K	6/64 L	1/64 M	2/64 N
4/64 P	2/64 Q	6/64 R	6/64 S	4/64 T	4/64 V
1/64 W	2/64 Y	3/64 stop			

This distribution has the disadvantage of giving two basic residues for every acidic residue. In addition, six times as much R, S, and L as W or M occur. If five codons are synthesized with this distribution, sequences encoding five Rs are 7776-times more abundant than sequences encoding five Ws. To have W-W-W-W-W present at detectable levels, we must have R-R-R-R-R present in 7776-fold excess.

Consider the distribution of amino acids encoded by one codon in a population of vgDNA. Let Abun(x) be the abundance of DNA sequences coding for amino acid x; Abun(x) is uniquely defined by the distribution of nts at each base of the codon. For any distribution, there will be a most-favored amino acid (mfaa) with abundance

Abun(mfaa) and a least-favored amino acid (lfaa) with abundance Abun(lfaa). We seek the nt distribution that allows all twenty amino acids and that yields the largest ratio Abun(lfaa)/Abun(mfaa) subject to two constraints. First, the abundances of acidic and basic amino acids should be equal lest we bias the PBDs toward a particular charge. Second, the number of stop codons should be kept as low as possible. Thus only nt distributions that yield Abun(E)+Abun(D) = Abun(R)+Abun(K) are considered, and the function maximized is:

((1-Abun(stop)) (Abun(lfaa)/Abun(mfaa))).

We have simplified the search for an optimal nt distribution by limiting the third base to T or G; C or G at the third base would be equivalent. All amino acids are possible and the number of accessible stop codons is reduced because TGA and TAA codons are eliminated. The amino acids F, Y, C, H, N, I, and D require T at the third base while W, M, Q, K, and E require G. Thus we use an equimolar mixture of T and G at the third base.

A computer program, written as part of the present invention and named "Find Optimum vgCodon" (See Table 9), varies the composition at bases 1 and 2, in steps of 0.05, and reports the composition that gives the largest value of the quantity ((Abun(lfaa)/Abun(mfaa) (1-Abun(stop)))). A vg codon is symbolically defined by the nt distribution at each base:

	T	C	A	G
base #1 =	t1	c1	a1	g1
base #2 =	t2	c2	a2	g2

base #3 = t3 c3 a3 g3

$$t1 + c1 + a1 + g1 = 1.0$$
$$t2 + c2 + a2 + g2 = 1.0$$
$$t3 = g3 = 0.5, \quad c3 = a3 = 0.$$

The variation of the quantities t1, c1, a1, g1, t2, c2, a2, and g2 is subject to the constraint that Abun(E)+Abun(D) equals Abun(K)+Abun(R);

$$Abun(E)+Abun(D) = g1*a2$$
$$Abun(K)+Abun(R) = a1*a2/2 + c1*g2 + a1*g2/2$$

$$g1*a2 = a1*a2/2 + c1*g2 + a1*g2/2$$

Solving for g2, we obtain

$$g2 = (g1*a2 - 0.5*a1*a2)/(c1 + 0.5*a1) \quad .$$

In addition,

$$t1 = 1 - a1 - c1 - g1$$
$$t2 = 1 - a2 - c2 - g2 \quad .$$

We vary a1, c1, g1, a2, and c2 and then calculate t1, g2, and t2. Initially, variation is in steps of 5%. Once an approximately optimum distribution of nts is determined, the region is further explored with steps of 1%. The logic of this program is shown in Table 9. The optimum distribution is:

Optimum vgCodon

	T	C	A	G
base #1 =	0.26	0.18	0.26	0.30
base #2 =	0.22	0.16	0.40	0.22
base #3 =	0.5	0.0	0.0	0.5

and yields DNA molecules encoding each type amino acid with the abundances shown in Table 10.

The computer that controls a DNA synthesizer, such as the Milligen 7500, can be programmed to synthesize any base of an oligo-nt with any distribution of nts by taking some nt substrates (e.g. nt phosphoramidites) from each of two or more reservoirs. Alternatively, nt substrates can be mixed in any ratios and placed in one of the extra reservoir for so called "dirty bottle" synthesis. Either of these methods amounts to specifying the nt distribution. The actual nt distribution obtained will differ from the specified nt distribution due to several causes, including: a) differential inherent reactivity of nt substrates, and b) differential deterioration of reagents. It is possible to compensate partially for these effects, but some residual error will occur. We denote the average discrepancy between specified and observed nt fraction as S_{err},

$$S_{err} = \text{square root} \left(\text{average} \left[(f_{obs} - f_{spec})/f_{spec} \right] \right)$$

were f_{obs} is the amount of one type of nt found at a base and f_{spec} is the amount of that type of nt that was specified at the same base. The average is over all specified types of nts and over a number (e.g. 10 or 20) different variegated bases. By hypothesis, the

actual nt distribution at a variegated base will be within 5% of the specified distribution. Actual DNA synthesizers and DNA synthetic chemistry may have different error levels. It is the user's responsibility to determine S_{err} for the DNA synthesizer and chemistry employed by the user.

To determine the possible effects of errors in nt composition on the amino-acid distribution, we modified the program "Find Optimum vgCodon" in four ways:

1) the fraction of each nt in the first two bases is allowed to vary from its optimum value times (1 - S_{err}) to the optimum value times (1 + S_{err}) in seven equal steps (S_{err} is the hypothetical fractional error level entered by the user); the sum of nt fractions at one base always equals 1.0,

2) g2 is varied in the same manner as a2, i.e. we dropped the restriction that Abun(D)+Abun(E) = Abun(K)+Abun(R),

3) t3 and g3 are varied from 0.5 times (1 - S_{err}) to 0.5 times (1 + S_{err}) in three equal steps,

4) the smallest ratio Abun(1faa)/Abun(mfaa) is sought.

In actual experiments, we will direct the synthesizer to produce the optimum DNA distribution "Optimum vgCodon" given above. Incomplete control over DNA chemistry may, however, cause us to actually obtain the following distribution that is the worst that can be obtained if all nt fractions are within 5% of the amounts specified in "Optimum vgCodon". A

corresponding table can be calculated for any given S_{err} using the program "Find worst vgCodon within Serr of given distribution." given in Table 11.

Optimum vgCodon, worst 5% errors

	T	C	A	G
base #1 =	0.251	0.189	0.273	0.287
base #2 =	0.209	0.160	0.400	0.231
base #3 =	0.475	0.0	0.0	0.525

This distribution yields DNA encoding different amino acids at the abundances shown in Table 12.

If five codons are synthesized with reagents mixed so as to produce the nt-distribution "Optimum vgCodon", and if we actually obtained the nt-distribution "Optimum vgCodon, worst 5% errors", then DNA sequences encoding the mfaa at all of the five codons are about 277 times as likely as DNA sequences encoding the lfaa at all of the five codons; about 24% of the DNA sequences will have a stop codon in one or more of the five codons.

When five codons are synthesized using equimolar mixtures at bases 1 and 2, $(Abun(mfaa)/Abun(lfaa))^5 = 7776$. If we program the optimum nt distribution and come within 5%, then $(Abun(mfaa)/Abun(lfaa))^5 = 277$. The total number of different PBDs is unchanged, but the least-favored sequence is about 28 times more abundant. Detecting the least-favored amino-acid sequence when varying four residues with equimolar nts at each varied base requires as sensitive a separation system as does detecting the least-favored amino-acid

sequence when varying five residues with the optimized nt distribution.

By hypothesis, the distribution "Optimal vgCodon" is used in the second version of the second variegation of hypothetical example 2. The abundance of the DNA encoding each type of amino acid is, however, taken from the Table 12. The abundance of DNA encoding the parental amino acid sequence is:

Amount(parental seq.)

F24 G30 D34 E42 T47

= Abun(F) * Abun(G) * Abun(D) * Abun(E) * Abun(T)

= .0249 x .0663 x .0545 x .0602 x .0437

= 2.4×10^{-7}

Therefore, DNA encoding the PPBD sequence as well as very many related sequences will be present in sufficient quantity to be detected and we are assured that the process will be progressive.

We use the following procedure to determine whether a given level of variegation is practical:

1) from: a) the intended nt-distribution at each base of a variegated codon, and b) S_{err} (the error level in mixed DNA synthesis), calculate the abundances of DNA sequences coding for each amino acid and stop,

2) calculate the abundance of DNA encoding the PPBD sequence by multiplying the abundances of the parental amino acid at each variegated residue,

The abundances used in the procedure above are calculated from the worst distribution that is within S_{err} of the specified distribution. A variegation that ensures that the PPBD sequence can be recovered is practical. PPBD can be recovered if the abundance of PPBD-encoding DNA is larger than both $1/M_{ntv}$ and $1/C_{sensi}$. Preferably, the abundance of PPBD-encoding DNA is 3 to 10 times higher than both $1/M_{ntv}$ and $1/C_{sensi}$ to provide a margin of redundancy. M_{ntv} is the number of transformants that can be made from Y_{D100} DNA. With current technology Mntv is approximately 5×10^8, but the exact value depends on the details of the procedures adapted by the user. Improvements in technology that allow more efficient: a) synthesis of DNA, b) ligation of DNA, or c) transformation of cells will raise the value of M_{ntv}. C_{sensi} is the sensitivity of the affinity separation; improvements in affinity separation will raise C_{sensi}. If the smaller of M_{ntv} and Csensi is increased, higher levels of variegation may be used. For example, if C_{sensi} is 1 in 10^9 and M_{ntv} is 10^8, then improvements in C_{sensi} are less valuable than improvements in M_{ntv}.

A level of variegation that allows recovery of the PPBD has two properties:

1) we can not regress because the PPBD is available,

2) an enormous number of multiple changes related to the PPBD are available for selection and we are able to detect and benefit from these changes.

It is very unlikely that all of the variants will be worse than the PPBD; we require the presence of PPBD

at detectable levels to insure that all the sequences present are indeed related to PPBD.

The user must adjust the list of residues to be varied and levels of variegation at each residue until the calculated variegation is within the bounds set by M_{ntv} and C_{sensi}.

Preferably, we also consider the interactions between the sites of variegation and the surrounding DNA. If the method of mutagenesis to be used is replacement of a cassette, we consider whether the variegation will generate gratuitous restriction sites and whether they seriously interfere with the intended introduction of diversity. We reduce or eliminate gratuitous restriction sites by appropriate choice of variegation pattern and silent alteration of codons neighboring the sites of variegation. See the Detailed Example.

Sec. 14.1: Insertion of synthetic vgDNA into a Plasmids:

In the case of cassette mutagenesis, the restriction sites that were introduced when the gene for the inserted domain was synthesized are used to introduce the synthetic vgDNA into a plasmid or other OCV. Restriction digestions and ligations are performed by standard methods (AUSU87).

In the case of single-stranded-oligonucleotide-directed mutagenesis, synthetic vgDNA is used to create diversity in the vector (BOTS85).

Sec. 14.2: Transformation of cells:

The present invention is not limited to any one method of transforming cells with DNA. The following procedure is a modification of that of Maniatis (p250, MANI82). This procedure is only one example of how the necessary transformations may be performed. The procedure produces approximately $(V_c/25) \times 10^7$ or more transformants. The user picks a value for V_c, the initial volume of the cell culture, to provide the desired number of transformants. All water is triple distilled and is treated with activated charcoal for 24 hours.

1) culture E. coli in V_c ml of LB broth at 37°C until cell density reaches 5×10^7 to 7×10^7 cells/ml,

2) chill on ice for 65 minutes, centrifuge the cell suspension at 4000g for 5 minutes at 4°C,

3) discard supernatant; resuspend the cells in $V_c/3$ ml of an ice-cold, sterile solution of 60 mM $CaCl_2$,

4) chill on ice for 15 minutes, and then centrifuge at 4000g for 5 minutes at 4°C,

5) discard supernatant; resuspend cells in $2 \times V_c/25$ ml of ice-cold, sterile 60 mM $CaCl_2$; store cells at 4°C for from 10 minutes to 24 hours; transformation efficiency increases by about 4-fold in the first 24 hours and then returns to the original value.

6) add DNA in ligation or TE buffer to $V_c/250$ ml of cells; mix and store on ice for 30 minutes.

7) heat shock cells at 42°C for an appropriate amount of time,

8) add V_c/25 ml LB broth and incubate at 37°C for 1 hour,

9) plate cells on LB agar containing antibiotic,

10) harvest GPs in appropriate manner.

It is not necessary to isolate transformed cells between transformation and affinity separation. We prefer to have transformed cells at high concentration so that they can be plated densely on relatively few plates. For this purpose, steps (9) and (10) may be replaced with a procedure in which the cells in step (8) are further diluted with LB broth and the selecting antibiotic is added. In the case of ampicillin, lysis of sensitive cells occurs, and resistant cells are enriched by centrifugation at 2 to 3 h after addition of antibiotic.

One routinely obtains between 10^7 and 5×10^8 transformants/ug of CCC DNA. Ligation efficiency ranges form 0.1% for blunt-blunt insertions, to as much as 15% for sticky-sticky insertions. For large transformations, it may be desirable to purify DNA between ligation and transformation because unligated DNA is thought to compete with CCC DNA for entry into the competent cells. Only a small fraction of cells are competent, typically 0.1%. The heat shock has been optimized for transformation reactions carried out in a volume of 200 ul in a plastic Eppendorf tube; optimizing this step for larger volumes is possible.

This procedure requires up to 2ug DNA per 10^7 transformants.

Sec. 14.3: Growth of the GP(vgPBD) population :

The transformed cells are grown first under non-selective conditions that allow expression of plasmid genes and then selected to kill untransformed cells. Transformed cells are then induced to express the ospA-pbd gene at the appropriate level of induction, as determined in Sec. 10.1. The GPs carrying the IPBD are harvested by a method appropriate to the package.

A high level of diversity can be generated by in vitro variegated synthesis of DNA and this diversity can be maintained passively through several generations in an organism without positive selective pressure. Loss or reduction in frequency of deleterious mutations is advantageous for the purposes of the present invention. As we do not know how one might press E. coli or any other kind of cell to actively maintain diversity, we specify that the vgDNA must be used to prepare plasmids, that the plasmids are used to transform cells, and that the selection must be performed before more than a few generations elapse. Moreover, subdividing the variegated population before amplification in an organism by removing a small sample (less than 10%) for further work would result in loss of diversity; therefore, one should use all or most of the synthetic DNA and most or all of the transformed cells.

Sec. 15.: Isolation of GP(PBD)s with binding-to-target phenotypes :

The harvested packages are now enriched for the binding-to-target phenotype by use of affinity separation involving the target material immobilized on an affinity matrix. Packages that fail to bind to the target material are washed away. If the packages are bacteriophage or endospores, it may be desirable to include a bacteriocidal agent, such as azide, in the buffer to prevent bacterial growth. The buffers used in chromatography must include: a) any ions or other solutes needed to stabilize the target, and b) any ions or other solutes needed to stabilize the PBDs derived from the IPBD.

Sec. 15.1: Attaching the target material to a column:

Affinity column chromatography is the preferred method of affinity separation, but other affinity separation methods may be used. A variety of commercially available support materials for affinity chromatography are used. These include derivatized beads to which the target material is covalently linked, or non-derivatized material to which the target material adheres irreversibly.

Suppliers of support material for affinity chromatography include: Applied Protein Technologies Cambridge, MA; Bio-Rad Laboratories, Rockville Center, NY; Pierce Chemical Company, Rockford, IL. Target materials are attached to the matrix in accord with the directions of the manufacturer of each matrix preparation with consideration of good presentation of the target.

Sec. 15.2: Reducing selection due to non-specific binding:

We reduce non-specific binding of GP(PBD)s to the matrix that bears the target in two ways:

1) we treat the column with blocking agents such as genetically defective GPs or a solution of protein before the population of GP(vgPBD)s is chromatographed, and

2) we pass the population of GP(vgPBD)s over a matrix containing no target or a different target from the same class as the actual target prior to affinity chromatography.

Step (1) above saturates any non-specific binding that the affinity matrix might show toward wild-type GPs or proteins in general; step (2) removes components of our population that exhibit non-specific binding to the matrix or to molecules of the same class as the target. If the target were horse heart myoglobin, for example, a column supporting bovine serum albumin could be used to trap GPs exhibiting PBDs with strong non-specific binding to proteins. If cholesterol were the target, then a hydrophobic compound, such as p-tertiarybutylbenzyl alcohol, could be used to remove GPs displaying PBDs having strong non-specific binding to hydrophobic compounds. It is anticipated that PBDs that fail to fold or that are prematurely terminated will be non-specifically sticky. These sequences could outnumber the PBDs having desirable binding properties. Thus, the capacity of the initial column that removes indiscriminately adhesive PBDs should be greater (e.g. 5 fold greater) than the column that supports the target molecule.

Variation in the support material (polystyrene, glass, agarose, cellulose, etc.) in analysis of clones carrying SBDs is used to eliminate enrichment for packages that bind to the support material rather than the target.

Sec. 15.J: Eluting the column:

To separate the GP(PBD)s that carry PBDs that show actual binding to the target from GP(PBD)s that carry PBDs that do not actually show binding to the target, the population of GPs is applied to an affinity matrix under conditions compatible with the intended use of the binding protein and the population is fractionated by passage of a gradient of some solute over the column. The process enriches for PBDs having affinity for the target and for which the affinity for the target is least affected by the eluants used. The enriched fractions are those containing viable GPs that elute from the column at greater concentration of the eluant.

Any ions or cofactors needed for stability of PBDs (derived from IPBD) or target must be included in initial and elution buffers at appropriate levels. We first remove GP(PBD)s that do not bind the target by washing the matrix with the volume of the initial buffer required to bring the optical density (at 260 nm or 280 nm) back to base line plus one void volume (V_V), but not more than 5 V_V. The column is then eluted with a gradient of increasing: a) salt, b) [H+] (decreasing pH), c) neutral solutes, d) temperature (increasing or decreasing), or e) some combination of these factors. The solutes in each of the first three gradients have been found generally to weaken non-

covalent interactions between proteins and bound molecules. Salt is the most preferred solute for gradient formation in most cases. Other solutes that generally weaken non-covalent interaction between proteins and bound molecules may also be used. "Salt" includes solutions containing any or all of the following ionic species:

Na^+	K^-	Ca^{++}	Mg^{++}
NH_4^+	Li^+	Sr^{++}	Ba^{++}
Rb^+	Cs^+	Cl^-	Br^-
SO_4^{--}	NSO_4^-	PO_4^{---}	HPO_4^{--}
$H_2PO_4^-$	CO_3^{--}	HCO_3^-	Acetate
Citrate	Standard l-Amino Acids	Standard nucleotides	Guanidinium Cl

Other ionic or neutral solutes may be used. All solutes are subject to the necessity that they not kill the genetic packages. Because bacteria continue to metabolize during affinity separation, the choice of buffer components is more restricted for bacteria than for bacteriophage or spores. Neutral solutes, such as ethanol, acetone, ether, or urea, are frequently used in protein purification and are known to weaken non-covalent interactions between proteins and other molecules. Many of these species are, however, very harmful to bacteria and bacteriophage. Bacterial spores, on the other hand, are impervious to most neutral solutes. Several passes may be made through the steps in Sec. 15. Different solutes may be used in different analyses, salt in one, pH in the next, etc.

Sec. 15.4: Recovery of packages:

Recovery of packages that display binding to an affinity column may be achieved in several ways, including:

1) collect fractions eluted from the column with a gradient as described above; fractions eluting later in the gradient contain GPs more enriched for genes encoding PBDs with high affinity for the column,

2) elute the column with the target material in soluble form,

3) flood the matrix with a nutritive medium and grow the desired packages *in situ*,

4) remove parts of the matrix and use them to inoculate growth medium,

5) chemically or enzymatically degrade the linkage holding the target to the matrix so that GPs still bound to target are eluted, or

6) degrade the packages and recover DNA with phenol or other suitable solvent; the recovered DNA is used to transform cells that regenerate GPs.

It is possible to utilize combinations of these methods. It should be remembered that what we want to recover from the affinity matrix is not the GPs *per se*, but the information in them. Recovery of viable GPs is very strongly preferred, but recovery of

genetic material is essential. If cells, spores, or virions bind irreversibly to the matrix but are not killed, we can recover the information through in situ cell division, germination, or infection respectively. Proteolytic degradation of the packages and recovery of DNA is not preferred.

Although degradation of the bound GPs and recovery of genetic material is a possible mode of operation, inadvertent inactivation of the GPs is very deleterious. It is preferred that maximum limits for solutes that do not inactivate the GPs or denature the target or the column are determined. If the affinity matrices are expendable, one may use conditions that denature the column to elute GPs; before the target is denatured, a portion of the affinity matrix should be removed for possible use as an inoculum. As the GPs are held together by protein-protein interactions and other non-covalent molecular interactions, there will be cases in which the molecular package will bind so tightly to the target molecules on the affinity matrix that the GPs can not be washed off in viable form. This will only occur when very tight binding has been obtained. In these cases, methods (3) through (5) above can be used to obtain the bound packages or the genetic messages from the affinity matrix.

It is possible, by manipulation of the elution conditions, to isolate SBDs that bind to the target at one pH (pH_b) but not at another pH (pH_o). The population is applied at pH_b and the column is washed thoroughly at pH_b. The column is then eluted with buffer at pH_o and GPs that come off at the new pH are collected and cultured. Similar procedures may be used for other solution parameters, such as

temperature. For example, GP(vgPBD)s could be applied to a column supporting insulin. After eluting with salt to remove GPs with little or no binding to insulin, we elute with salt and glucose to liberate GPs that display PBDs that bind insulin or glucose in a competitive manner.

Sec. 15.5: Amplifying the Enriched Packages

Viable GPs having the selected binding trait are amplified by culture in a suitable medium, or, in the case of phage, infection into a host so cultivated. If the GPs have been inactivated by the chromatography, the OCV carrying the osp-pbd gene must be recovered from the GP, and introduced into a new, viable host.

Sec. 15.6: Determining whether further enrichment is needed:

The probability of isolating a GP with improved binding increases by C_{eff} with each separation cycle. Let N be the number of distinct amino-acid sequences produced by the variegation. We want to perform K separation cycles before attempting to isolate an SBD, where K is such that the probability of isolating a single SBD is 0.10 or higher.

K = the smallest integer>= $\log_{10}(0.10\ N)/\log_{10}(C_{eff})$

For example, if N were 1.0×10^7 and C_{eff} = $6.31 \times 10_2$, then log10(1.0 x 106)/log10(6.31 x 102) = 6.0000/2.8000 = 2.14. Therefore we would attempt to isolate SBDs after the third separation cycle. After only two separation cycles, the probability of finding

an SBD is $(6.31 \times 10^2)^2/(1.0 \times 10^7) = .04$ and attempting to isolate SBDs might be profitable.

Clonal isolates from the last fraction eluted in Sec. 15.3 containing any viable GPs, as well as clonal isolates obtained by culturing an inoculum taken from the affinity matrix, are cultured in a growth step that is similar to that described in Sec. 14.3. If K separation cycles have been completed, samples from a number, e.g. 32, of these clonal isolates are tested for elution properties on the (target) column. If none of the isolated, genetically pure GPs show improved binding to target, or if K cycles have not yet been completed, then we pool and culture, in a manner similar to the manner set forth in SEc. 14.3, the GPs from the last few fractions eluted (see Sec. 15.4) that contained viable GPs and from the GPs obtained by culturing an inoculum taken from the column matrix. We then repeat the enrichment procedure described in Sec. 15. This cyclic enrichment may continue N_{chrom} passes or until an SBD is isolated.

If one or more of the isolated GPs has improved retention on the (target) column, we determine whether the retention of the candidate SBDs is due to affinity for the target material as follows. A second column is prepared using a different support matrix with the target material bound at the optimal density. The elution volumes, under the same elution conditions as used previously (see Sec. 15.3), of candidate GP(SBD)s are compared to each other and to GP(PPBD of this round). If one or more candidate GP(SBD)s has a larger elution volume than GP(PPBD of this round), then we pick the GP(SBD) having the highest elution

volume and proceed to characterize the population (see Sec. 15.7). If none of the candidate GP(SBD)s has higher elution volume than GP(PPBD of this round), then we pool and culture, in a manner similar to the manner used previously (Sec. 15.3), the GPs from the last few fractions that contained viable GPs and the GPs obtained by culturing an inoculum taken from the column matrix. We then repeat the enrichment procedure of Sec. 15.

If all of the SBDs show binding that is superior to PPBD of this round, we pool and culture the GPs from the last fraction that contains viable GPs and from the inoculum taken from the column. This population is re-chromatographed at least one pass to fractionate further the GPs based on K_d.

If an RNA phage were used as GP, the RNA would either be cultured with the assistance of a helper phage or be reverse transcribed and the DNA amplified. The amplified DNA could then be sequenced or subcloned into suitable plasmids.

Sec. 15.7: Characterizing the Population:

We characterize members of the population showing desired binding properties by genetic and biochemical methods. We obtain clonal isolates and test these strains by genetic and affinity methods to determine genotype and phenotype with respect to binding to target. For several genetically pure isolates that show binding, we demonstrate that the binding is caused by the artificial chimeric gene by excising the osp-sbd gene and crossing it into the parental GP. We also ligate the deleted backbone of each GP from which

the osp-sbd is removed and demonstrate that each backbone alone cannot confer binding to the target on the CP. We sequence the osp-sbd gene from several clonal isolates. Primers for sequencing are chosen from the DNA flanking the osp-ppbd gene or from parts of the osp-ppbd gene that are not variegated.

Sec. 15.8: Testing of binding affinity:

For one or more clonal isolates, we subclone the sbd gene fragment, without the osp fragment, into an expression vector such that each SBD can be produced as a free protein. Because numerous unique restriction sites were built into the inserted domain, it is easy to subclone the gene at any time. Each SBD protein is purified by normal means, including affinity chromatography. Physical measurements of the strength of binding are then made on each free SBD protein by one of the following methods: 1) alteration of the Stokes radius as a function of binding of the target material, measured by characteristics of elution from a molecular sizing column such as agarose, 2) retention of radiolabeled binding protein on a spun affinity column to which has been affixed the target material, or 3) retention of radiolabeled target material on a spun affinity column to which has been affixed the binding protein. The measurements of binding for each free SBD are compared to the corresponding measurements of binding for the PPBD.

In each assay, we measure the extent of binding as a function of concentration of each protein, and other relevant physical and chemical parameters such as salt concentration, temperature, pH, and prosthetic group concentrations (if any).

In addition, the SBD with highest affinity for the target from each round is compared to the best SBD of the previous round (IPBD for the first round) and to the IPBD (second and later rounds) with respect to affinity for the target material. Successive rounds of mutagenesis and selection-through-binding yield increasing affinity until desired levels are achieved.

If we find that the binding is not yet sufficient, we must decide which residues to vary next (see Sec. 16.0). If the binding is sufficient, then we now have a expression vector bearing a gene encoding the desired novel binding protein.

Sec. 15.9: Other Affinity Separation Means:

FACs may be used to separate GPs that bind fluorescent labeled target with the optimized parameters determined in Part II. We discriminate against artifactual binding to the fluorescent able by using two or more different dyes, chosen to be structurally different. GPs isolated using target labeled with a first dye are cultured. These GPs are then tested with target labeled with a second dye.

Electrophoretic affinity separation uses unaltered target so that only other ions in the buffer can give rise to artifactual binding. Artifactual biding to the gel material gives rise to retardation independent of field direction and so is easily eliminated. A variegated population of GPs will have a variety of charges. The following 2D electrophoretic procedure accommodates this variation in the population.

First the variegated population of GPs is electrophoresed in a gel that contains no target material. The electrophoresis continues until the GPs are distributed along the length of the lane. The gels described by Sewer for phage are very low in agarose and lack mechanical stability. The target-free lane in which the initial electrophoresis is conducted is separate from a square of gel that contains target material by a removable baffle. After the first pass, the baffle is removed and a second electrophoresis is conducted at right angles to the first. GPs that do not bind target migrate with unaltered mobility while GPs that do bind target will separate from the majority that do not bind target. A diagonal line of non-binding GPs will form. This line is excised and discarded. Other parts of the gel are dissolved and the GPs cultured.

Sec. 16.0: The Next Variegation Cycle:

We now consider which residues of the PBD should be varied in the next variegation cycle. The general rule is to preserve as much accumulated information as possible. If the level of variegation in the previous variegation cycle was correctly chosen, then the amino acids selected to be in the residues just varied are the ones best determined. The environment of other residues has changed, so that it is appropriate to vary them again. Because there are always more residues in the principal (Sec. 13.1.1) and secondary sets (Sec. 13.1.2) than can be varied simultaneously, we start by picking residues that either have never been varied (highest priority) or that have not been varied for one or more cycles. If we find that

varying all the residues except those varied in the previous cycle does not allow a high enough level of diversity, then residues varied in the previous cycle might be varied again. For example, if M_{ntv} (the number of independent transformants that can be produced from Y_{D100} of DNA) and C_{sensi} (the sensitivity of the affinity separation) were such that seven residues could be varied, and if the principal and secondary sets contained 13 residues, we would always vary seven residues, even though that implies varying some residue twice in a row. In such cases, we would pick the residues just varied that contain the amino acids of highest abundance in the variegated codons used.

It is the accumulation of information that allows the process to select those protein sequences that produce binding between the SBD and the target. Some interfaces between proteins and other molecules involve twenty or more residues. Complete variation of twenty residues would generate 10^{26} different proteins. By dividing the residues that lie close together in space into overlapping groups of five to seven residues, we can vary a large surface but never need to test more than 10^7 to 10^9 candidates at once, a savings of 10^{19} to 10^{17} fold. The power of selection with accumulation of information is well illustrated in Chapter 3 of DAWK86.

Having picked the residues to vary, we again set the range of variegation for each residue according to the principles set forth in 13.2, design the vgDNA encoding the desired mutants (Sec. 13.3), clone the vgDNA into GPs (Sec. 14), and select-by-binding-to-target those GPs bearing SBDs (Sec. 15).

Sec. 17.0: OTHER CONSIDERATIONS:

Sec. 17.1: Joint selections:

One may modify the affinity separation of the method described to select a molecule that binds to material A but not to material B. One needs to prepare two selection columns, one with material A and the other with material B. The population of genetic packages is prepared in the manner described, but before applying the population to A, one passes the population over the B column so as to remove those members of the population that have high affinity for B ("reverse affinity chromatography"). In the preceding specification, the initial column supported some other molecule simply to remove GP(PSD)s that displayed PBDs having indiscriminate affinity for surfaces.

It may be necessary to amplify the population that does not bind to B before passing it over A. Amplification would most likely be needed if A and B were in some ways similar and the PPBD has been selected for having affinity for A. The optimum order of interactions might be determined empirically.

For example, to obtain an SBD that binds A but not B, three columns could be connected in series: a) a column supporting some compound, neither A nor B, or only the matrix material, b) a column supporting B, and c) a column supporting A. A population of GP(vgPBD)s is applied to the series of columns and the columns are washed with the buffer of constant ionic strength that is used in the application. The columns

are uncoupled, and the third column is eluted with a gradient to isolate GP(PBD)s that bind A but not B.

One can also generate molecules that bind to both A and B. In this case we can use a 3D model and mutate one face of the molecule in question to get binding to A. One can then mutate a different face to produce binding to B. When an SBD binds at least somewhat to both A and B, one can mutate the chain by Diffuse Mutagenesis to refine the binding and use a sequential joint selection for binding to both A and B.

The materials A and B could be proteins that differ at only one or a few residues. For example, A could be a natural protein for which the gene has been cloned and B could be a mutant of A that retains the overall 3D structure of A. SBDs selected to bind A but not B must bind to A near the residues that are mutated in B. If the mutations were picked to be in the active site of A (assuming A has an active site), then an SBD that binds A but not B will bind to the active site of A and is likely to be an inhibitor of A.

To obtain a protein that will bind to both A and B, we can, alternatively, first obtain an SBD that binds A and a different SBD that binds B. We can then combine the genes encoding these domains so that a two-domain single-polypeptide protein is produced. The fusion protein will have affinity for both A and B because one of its domains binds A and the other binds B.

One can also generate binding proteins with affinity for both A and B, such that these materials will compete for the same site on the binding protein. We guarantee competition by overlapping the sites for A and B. Using the procedures of the present invention, we first create a molecule that binds to target material A. We then vary a set of residues defined as: a) those residues that were varied to obtain binding to A, plus b) those residues close in 3D space to the residues of set (a) but that are internal and so are unlikely to bind directly to either A or B. Residues in set (b) are likely to make small changes in the positioning of the residues in set (a) such that the affinities for A and B will be changed by small amounts. Members of these populations are selected for affinity to both A and B.

Sec. 17.2: Selection for non-binding:

The method of the present invention can be used to select proteins that do not bind to selected targets. Consider a protein of pharmacological importance, such as streptokinase, that is antigenic to an undesirable extent. We can take the pharmacologically important protein as IPBD and antibodies against it as target. Residues on the surface of the pharmacologically important protein would be varicgated and GP(PBD)s that do not bind to an antibody column would be collected and cultured. Surface residues may be identified in several ways, including: a) from a 3D structure, b) from hydrophobicity considerations, or c) chemical labeling. The 3D structure of the pharmacologically important protein remains the preferred guide to picking residues to vary, except now we pick residues

that are widely spaced so that we leave as little as possible of the original surface unaltered.

Destroying binding frequently requires only that a single amino acid in the binding interface be changed. If polyclonal antibodies are used, we face the problem that all or most of the strong epitopes must be altered in a single molecule. Preferably, one would have a set of monoclonal antibodies, or a narrow range of antibody species. If we had a series of monoclonal antibody columns, we could obtain one or more mutations that abolish binding to each monoclonal antibody. We could then combine some or all of these mutations in one molecule to produce a pharmacologically important protein recognized by none of the monoclonal antibodies. Such mutants must be tested to verify that the pharmacologically interesting properties have not be altered to an unacceptable degree by the mutations.

Typically, polyclonal antibodies display a range of binding constants for antigen. Even if we have only polyclonal antibodies that bind to the pharmacologically important protein, we may proceed as follows. We engineer the pharmacologically important protein to appear on the surface of a replicable GP. We introduce mutations into residues that are on the surface of the pharmacologically important protein or into residues thought to be on the surface of the pharmacologically important protein so that a population of GPs is obtained. Polyclonal antibodies are attached to a column and the population of GPs is applied to the column at low salt. The column is eluted with a salt gradient. The GPs that elute at the lowest concentration of salt are those which bear

pharmacologically important proteins that have been mutated in a way that eliminates binding to the antibodies having maximum affinity for the pharmacologically important protein. The GPs eluting at the lowest salt are isolated and cultured. The isolated SBD becomes the PPBD to further rounds of variegation so that the antigenic determinants are successively eliminated.

Sec. 17.1: Selection of PBDs for retention of structure:

Let us take an SBD with known affinity for a target as PPBD to a variegation of a region of the PBD that is far from the residues that were varied to create the SBD. We can use the target as an affinity molecule to select the PBDs that retain binding for the target, and that presumably retain the underlying structure of the IPBD. The variegations in this case could include insertions and deletions that are likely to disrupt the IPBD structure. We could also use the IPBD and AfM(IPBD) in the same way.

For example, if IPBD were BPTI and AfM(BPTI) were trypsin, we could introduce four or five additional residue after residue 26 and select GPs that display PBDs having specific affinity for AfM(BPTI). Residue 26 is chosen because it is in a turn and because it is about 25 A from K15, a key amino acid in binding to trypsin.

The underlying structure is most likely to be retained if insertions or deletions are made at loops or turns.

Sec. 17.4: Created binding proteins not unique:

For each target, there are a large number of SBDs that may be found by the method of the present invention. The process relies on a combination of protein structural considerations, probabilities, and targeted mutations with accumulation of information. To increase the probability that some PBD in the population will bind to the target, we generate as large a population as we can conveniently subject to selection-through-binding in one experiment. Key questions in management of the method are "How many transformants can we produce?", and "How small a component can we find through selection-through-binding?". Geneticists routinely find mutations with frequencies of one in 10^{10} using simple, powerful selections; we experimentally determine the sensitivity of our procedure. The optimum level of variegation is determined by the maximum number of transformants and the selection sensitivity, so that for any reasonable sensitivity we may use a progressive process to obtain a series of proteins with higher and higher affinity for the chosen target material. Enrichments of 1000-fold by a single pass of elution from an affinity plate have been demonstrated (SMIT85). Three rounds of such enrichment could produce 10^9-fold enrichment, and additional rounds may be added if necessary.

Use of different variation schemes can yield different binding proteins. For any given target, there is a large plurality of proteins that will bind to it. Thus, if one binding protein turns out to be unsuitable for some reason (e.g. too antigenic), the procedure can be repeated with different variation

parameters. For example, one might choose different residues to vary or pick a different nt distribution at variegated codons so that a new distribution of amino acids is tested at the same residues. Even if the same principal set of residues is used, one might obtain a different SBD if the order in which one picks subsets to be varied is altered.

Sec. 17.5: Other modes of mutagenesis possible:

The modes of creating diversity in the population of GPs discussed herein are not the only modes possible. Any method of mutagenesis that preserves at least a large fraction of the information obtained from one selection and then introduces other mutations in the same domain will work. The limiting factors are the number of independent transformants that can be produced and the amount of enrichment one can achieve through affinity separation. Therefore the preferred embodiment uses a method of mutagenesis that focuses mutations into those residues that are most likely to affect the binding properties of the PBD and are least likely to destroy the underlying structure of the IPBD.

Other modes of mutagenesis might allow other GPs to be considered. For example, the bacteriophage lambda is not a useful cloning vehicle for cassette mutagenesis because of the plethora of restriction sites. One can, however, use single-stranded-oligo-nt-directed mutagenesis on lambda without the need for unique restriction sites. No one has used single-stranded-oligo-nt-directed mutagenesis to introduce the high level of diversity called for in the present invention, but if it is possible, such a method would allow use of phage with large genomes.

Example 1

BPTI-Derived Binding Protein for HHMb; Displayed by M13 Phage

Presented below is a hypothetical example of a protocol for developing a new binding molecule derived from BPTI with affinity for horse heart myoglobin (HHMb) using the common E. coli bacteriophage M13 as genetic package. It will be understood that some further optimization, in accordance with the teachings herein, may be necessary to obtain the desired results. Possible modifications in the preferred method are discussed immediately following various steps of the hypothetical example.

By hypothesis, we set the following technical capabilities:

Y_{DQ} 500 ng/synthesis of ssDNA 100 bases long,
10 ug/synthesis of ssDNA 60 bases long,
1 mg/synthesis of ssDNA 20 bases long.

M_{DNA} 100 bases

Y_{pl} 1 mg/l

L_{ef} 0.1 % for blunt-blunt,
4 % for sticky-blunt,
11 % for sticky-sticky.

M_{ntv} 5×10^8

C_{eff}	900-fold enrichment
C_{sensi}	1 in 4 x 10^8
N_{chrom}	10 passes
S_{err}	0.05

Example 1, Part I

In this example, we will use M13 as a replicable GP and BPTI as IPBD. The considerations that lead to these choices are discussed. In Part I, we are concerned only with getting BPTI displayed on the outer surface of an M13 derivative. Variable DNA may be introduced in the osp-ipbd gene, but not within the region that codes for the trypsin-binding region of BPTI. Once BPTI is displayed on the M13 outer surface of an M13 derivative, we proceed to Part II to optimize the affinity separation procedures.

We consider various GPs and, for this example, choose a filamentous bacteriophage of E. coli, M13. We prefer phage over vegetative bacterial cells because phage are much less metabolically active. We prefer phage over spores because the molecular mechanisms of the virion formation and 3D structure of the virion are much better understood than are the corresponding processes of spore formation and structures of spores.

M13 is a very well studied bacteriophage, widely used for DNA sequencing and as a genetic vector; it is a typical member of the class of filamentous phages. The relevant facts about M13 and other phages that will

allow us to choose among phages are cited in Sec. 1.3.1.

Compared to other bacteriophage, filamentous phage in general are attractive and M13 in particular is especially attractive because:

1) the 3D structure of the virion is known,

2) the processing of the coat protein is well understood,

3) the genome is expandable,

4) the genome is small,

5) the sequence of the genome is known,

6) the virion is physically resistant to shear, heat, cold, guanidinium Cl, low pH, and high salt,

7) the phage is a sequencing vector so that sequencing is especially easy, and

8) antibiotic-resistance genes have been cloned into the genome with predictable results (HINE80).

Other criteria listed in Sec. 1.0 and 1.3 of the are also satisfied: M13 is easily cultured and stored (FRIT85), each infected cell yielding 100 to 1000 M13 progeny after infection. M13 has no unusual or expensive media requirements and is easily harvested and concentrated (SALI64, FRIT85). M13 is stable toward physical agents: temperature (10% of phage survive 30 minutes at 85°C), shear (Waring blender does

not kill), desiccation (not applicable), radiation (not applicable), age (stable for years).

M13 is stable toward chemicals: pH (< 2.2 (SMIT85)), surface active agents: not applicable, chaotropes (guanidinium HCl = 6.0 M), ions (no specific sensitivities), organic solvents (ether and other organic solvents are lethal (MARV78)), proteases (not applicable, HMMb not a protease). M13 is not known to be sensitive to other enzymes.

M13 genome is 6423 b.p. and the sequence is known (SCHA78). Because the genome is small, cassette mutagenesis is practical on RF M13 (AUSU87), as is single-stranded oligo-nt directed mutagenesis (FRIT85). M13 is a plasmid and transformation system in itself, and an ideal sequencing vector. M13 can be grown on Rec$^-$ strains of E. coli. The M13 genome is expandable (MESS78, FRIT85). M13 confers no advantage, but doesn't lyse cells. The sequence of gene VIII is known, and the amino acid sequence can be encoded on a synthetic gene, using lacUV5 promoter and used in conjunction with the LacI^q repressor. The lacUV5 promoter is induced by IPTG. Gene VIII protein is secreted by a well studied process and is cleaved between A23 and A24. Residues 18, 21, 22, and 23 of gene VIII protein control cleavage. Mature gene VIII protein makes up the sheath around the circular ssDNA. The 3D structure of f1 virion is known at medium resolution: the amino terminus of gene VIII protein is on surface of the virion. No fusions to M13 gene VIII protein have been reported. The 2D structure of M13 coat protein is implicit in the 3D structure. Mature M13 gene VIII protein has only one domain. There are four minor proteins: gene III, VI, VII, and IX. Each

of these minor proteins is present in about 5 copies per virion and is related to morphogenesis or infection. The major coat protein is present in more than 2500 copies per virion.

Although no fusions of M13 gene VIII to other genes have been reported, knowledge of the virion 3D structure makes attachment of IPBD to the amino terminus of mature M13 coat protein (M13 CP) quite attractive (See Sec. 1.3.2). Should direct fusion of BPTI to M13 CP fail to cause BPTI to be displayed on the surface of M13, we will vary part of the BPTI sequence and/or insert short random DNA sequences between BPTI and M13 CP (Sec. 1.3.4).

Smith (SMIT85) and de la Cruz et al. (CRU288) have shown that insertions into gene III cause novel protein domains to appear on the virion outer surface. If BPTI can not be made to appear on the virion outer surface by fusing the bpti gene to the m13cp gene, we will fuse bpti to gene III either at the site used by Smith and by de la Cruz et al. or to one of the termini. We will use a second, synthetic copy of gene III so that some unaltered gene III protein will be present.

The gene VIII protein is chosen as OSP because it is present in many copies and because its location and orientation in the virion are known. Note that any uncertainty about the azimuth of the coat protein about its own alpha helical axis is unimportant: the amino terminus is exposed for all azimuths.

The 3D model of f1 indicates strongly that fusing BPTI to the amino terminus of M13 CP is more likely to yield a functional protein than any other fusion site.

(See Sec. 1.3.3).

The amino-acid sequence of M13 pre-coat (SCHA78), called AA_seq1, is

AA_seq1

```
          1    1    2    2    3    3    4    4    5
     5    0    5    0    5    0    5    0    5    0
MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAFNSLQASATEYIGYAWA

     5    6    6    7  7
     5    0    5    0  3
MVVVIVGATIGIKLFKKFTSKAS
```

The single-letter codes for amino acids and the codes for ambiguous DNA are given in Table 1. The best site for inserting a novel protein domain into M13 CP is after A23 because SP-I cleaves the precoat protein after A23, as indicated by the arrow. Proteins that can be secreted will appear connected to mature M13 CP at its amino terminus. Because the amino terminus of mature M13 CP is located on the outer surface of the virion, the introduced domain will be displayed on the outside of the virion.

BPTI is chosen as IPBD of this example (See Sec. 2.1) because it meets or exceeds all the criteria: it is a small, very stable protein with a well known 3D structure. Marks et al. (MARK86) have shown that a fusion of the phoA signal peptide gene fragment and DNA coding for the mature form of BPTI caused native BPTI to appear in the periplasm of E. coli, demonstrating that there is nothing in the structure of BPTI to prevent its being secreted.

Marks et al. (MARK87) also showed that the structure of BPTI is stable even to the removal of one

of the cystine bridges. They did this by replacing both C14 and C38 with either two alanines or two threonines. The C14/C38 cystine bridge that Marks et al. removed is the one very close to the scissile bond in BPTI; surprisingly, both mutant molecules functioned as trypsin inhibitors. This indicates that BPTI is redundantly stable and so is likely to fold into approximately the same structure despite numerous surface mutations. Using the knowledge of homologues, vide infra, we can infer which residues must not be varied if the basic BPTI structure is to be maintained.

The 3D structure of BPTI has been determined at high resolution by X-ray diffraction (HUBE77, MARQ83, WLOD84, WLOD87a, WLOD87b), neutron diffraction (WLOD84), and by NMR (WAGN87). In one of the X-ray structures deposited in the Brookhaven Protein Data Bank, "6PTI", there was no electron density for A58, indicating that A58 has no uniquely defined conformation. Thus we know that the carboxy group does not make any essential interaction in the folded structure. The amino terminus of BPTI is very near to the carboxy terminus. Goldenberg and Creighton reported on circularized BPTI and circularly permuted BPTI (GOLD83). Some proteins homologous to BPTI have more or fewer residues at either terminus.

BPTI has been called "the hydrogen atom of protein folding" and has been the subject of numerous experimental and theoretical studies (STAT87, SCHW87, GOLD83, CHAZ83).

BPTI has the added advantage that at least 32 homologous proteins are known, as shown in Table 13. A tally of ionizable groups is shown in Table 14 and the

composite of amino acid types occurring at each residue is shown in Table 15.

BPTI is freely soluble and is not known to bind metal ions. BPTI has no known enzymatic activity. BPTI binds to trypsin, $K_d = 6.0 \times 10^{-14}$ M (TSCH87). BPTI is not toxic. If K15 of BPTI is changed to L, there is no measurable binding between the mutant BPTI and trypsin (TSCH87).

Stereo Figure 7 shows the alpha carbons of BPTI plus the side groups of conserved residues; all four atoms of conserved glycines are shown. All of the conserved residues are buried; of the seven fully conserved residues only G37 has noticeable exposure. The solvent accessibility of each residue in BPTI is given in Table 16 which was calculated from the entry "6PTI" in the Brookhaven Protein Data Bank with a solvent radius of 1.4 A, the atomic radii given in Table 7, and the method of Lee and Richards (LEEB71). Each of the 51 non-conserved residues can accommodate two or more kinds of amino acids. By independently substituting at each residue only those amino acids already observed at that residue, we could obtain approximately 7×10^{12} different amino acid sequences, most of which will fold into structures very similar to BPTI.

BPTI will be useful as a IPBD for macromolecules. (See Sec. 2.1.1) BPTI and BPTI homologues bind tightly and with high specificity to a number of enzymes.

BPTI is strongly positively charged except at very high pH, thus BPTI is useful as IPBD for targets that are not also strongly positive under the conditions of

intended use (see Sec. 2.1.2). There exist homologues of BPTI, however, having quite different charges (viz. SCI-III from Bombyx mori at -7 and the trypsin inhibitor from bovine colostrum at -1). Once a derivative of M13 is found that displays BPTI on its surface, the sequence of the BPTI domain can be replaced by one of the homologous sequences to produce acidic or neutral IPBDs.

BPTI is not an enzyme (See Sec. 2.1.3). BPTI is quite small; if this should cause a pharmacological problem, two or more BPTI-derived domains may be joined as in the human BPTI homologue that has two domains.

A derivative of M13 is the preferred OCV. (See Sec. 3). Wild-type M13 does not confer any resistances on infected cells; M13 is a pure parasite. A "phagemid" is a hybrid between a phage and a plasmid, and is used in this invention. Double-stranded plasmid DNA isolated from phagemid-bearing cells is denoted by the standard convention, e.g. pXY24. Phage prepared from these cells would be designated XY24. Phagemids such as Bluescript K/S (sold by Stratagene) are not suitable for our purposes because Bluescript does not contain the full genome of M13 and must be rescued by coinfection with competent wild-type M13. Such coinfections will likely lead to genetic recombination yielding heterogeneous phage unsuitable for the purposes of the present invention.

It is also well known that plasmids containing the ColE1 origin of replication can be greatly amplified if protein synthesis is halted in a log-phase culture. Protein synthesis can be halted by addition of chloramphenicol or other agents (MANI82).

The bacteriophage M13 bla 61 (ATCC 37039) is derived from wild-type M13 through the insertion of the beta lactamase gene (HINE80). This phage contains 8.13 kb of DNA. M13 bla cat 1 (ATCC 37040) is derived from M13 bla 61 through the additional insertion of the chloramphenicol resistance gene (HINE80); M13 bla cat 1 contains 9.88 kb of DNA. Although neither of these variants of M13 contains the ColE1 origin of replication, either could be used as a starting point to construct a usable cloning vector for the present example.

The OCV for the current example is constructed by a process illustrated in Figure 8. A brief description of all the plasmids and phagemids constructed for this Example is found in Table 17.

For ss oligo-nt site-directed mutagenesis, multiple primers lead to higher efficiency. Three non-mutagenic primers are used :

```
5'(2326) GGC GGC TCT GAG GGT GGC GGT (2352) 3' wtM13
     3'  ccg ccg aga ctc cca ccg cca   5'  olig=24 ,


5'(4854) GCT GCT GGC TCT GAG CGC GGC (4875) 3' wtM13
     3'  cga cga ccg aga gtc gcg ccg   5'  olig=25 ,
```

and

```
5'(3451) CCG GTG AGC GTG GGT CTC GCG (3431) 3'
     3'  ggc cac tcg cac cca gag cgc   5'  olig=26 .
```

Olig=24 is complementary to a segment near the end of M13 gene III and olig=25 is complementary to part of

M13 gene IV (SCHA78). Oligo#26 is part of the amp^R gene from pBR322 (MANI82, Appendix B); the numbers shown refer to pBR322 base pair numbers. Note that pLG2 and its derivatives carry the anti-sense strand of the amp^R gene in the + DNA strand. The segments are picked to be high in GC content and to divide the pLG7 genome into several segments of approximately equal length.

The genetic engineering procedures needed to construct the OCV are standard. All restriction digests use commercially available enzymes and are carried out under conditions recommended by the supplier. All restriction fragments of DNA are purified by HPLC or electrophoresis from agarose gels as described elsewhere in the present invention. Competent E. coli are preferably prepared by a modified version of the procedure of Maniatis (MANI82) given in the generic detail section. M13 and its engineered derivatives are infected into E. coli strain PE384 (F^+,Rec^-,Sup^+,Amp^S). Plasmid DNA of M13 derivatives is transformed into E. coli strain PE383(F^-,Rec^-,Sup^+,Amp^S) so that we avoid multiple infections that might arise once phage are produced. Isolation of M13 phage is by the procedure of Salivar et al. (SALI64); isolation of replicative form (RF) M13 is by the procedure of Jazwinski et al. (JAZW75a and JAZW75b). Isolation of plasmids containing the ColE1 origin of replication is by the method of Maniatis (MANI82).

DNA sequencing is by the method of Sanger (AUSU87). Virions of M13 derivatives contain circular ss DNA that is called the viral + strand. Base numbers are assigned from an agreed origin and in ascending order in the 5'-to-3' direction of the viral + strand. Conventionally, this DNA is drawn with the 5'-to-3'

direction clockwise and corresponding to increasing base number. In relation to the genomes of M13 derivatives, we will use "up" or "above" to mean higher base number or further along in clockwise direction. Similarly "down" and "below" will mean lower base number or further along in the counterclockwise direction. To determine the base sequence of part of an M13 derivative, one needs a sequencing primer that is complementary to a region above and within about 100 bases of the region to be sequenced. Because the OCV is constructed from parts of M13mp18, parts of pBR322, and synthetic DNA, the sequence of flanking regions is always known.

We pick the amp^R gene from pBR322 as a convenient antibiotic resistance gene. Another resistance gene, such as kanamycin, could be used. (The New England BioLabs 1988/89 catalogue contains a genetic map of pBR322 on page 106.) The plasmid pBR322 also contains the ColE1 origin of replication. The restriction sites Acc I at 2246 and Aat II at 4286 are the most convenient places to cut pBR322 to obtain both an intact amp^R gene and the ColE1 origin of replication with ends suitable for ligation to other DNA.

The plasmid pBR322 contains a unique AlwN I site at base 2886 that is between the amp^R gene and ori. There is a unique AlwN I site in M13mp18 at base 2187. When the Acc I-to-Aat II fragment of pBR322 is ligated into M13mp18, there will be two AlwN I sites and no easy way to excise the amp^R gene. Thus we convert the AlwN I site of pBR322 into an Xba I site that will be unique in all the DNA constructs of the present example. The two oligo-nts:

```
5'     ccgaTCTAGActagtcgCCA   3'  olig:60
3' GGTggctAGATCTgatcagc       5'  olig:61
     |XbaI|
```

are synthesized by standard methods and annealed. The AlwN I site at base 2886 in pBR322 has the sequence 5'-CAGCCACTG - 3'. Plasmid pBR322 is cut with AlwN I and mixed with the synthetic ds DNA and ligated. Cells are transformed and selected for tetracycline resistance. Tetracycline resistant colonies are screened for the correct insert by restriction digestion with Xba I that cuts the correct construction but not pBR322. The correct construction is called pLG322. Plasmid pLG322 differs from pBR322 only by the replacement of the AlwN I site with an Xba I site.

The plasmid pLG322 contains a second Acc I restriction site at base 651 so that digestion of pLG322 with Aat II and Acc I yields three fragments, one of about 2041 bases (that we want), one of about 728 bases, and one of about 1600 bases. To facilitate isolation of the 2041-base fragment, we also digest pLG322 with Stv I that cuts at base 1369. The Stv I cut reduces the 1600-base fragment to two fragments of about 700 and about 900 bases each. We purify the 2041-nt fragment by HPLC or agarose gel electrophoresis.

M13mp18, sold by New England BioLabs, contains neither Aat II nor Acc I sites. Therefore we insert an adaptor that allows us to insert the Aat II-to-Acc I fragment of pLG322 that carries the amp^R gene and the ColE1 origin of replication into a desirable place in M13mp18. M13mp13 contains a lacUV5 promoter and a lacZ gene that are not useful to the purposes of the present invention. By cutting M13mp18 with Ava II at the

unique site at 5914 and with Bsu36 I at the unique site at 6508 and discarding the approximately 600 intervening base pairs, we eliminate all recognition sites of the enzymes shown in Table 18 from M13mp18.

M13mp18 itself is not cut by the enzymes listed in Table 19. Among the enzymes in Tables 18 and 19, those listed in Table 20 have recognition sites within the Acc I-to-Aat II fragment of pLG322 that contains the amp^R gene and the ColE1 origin of replication.

Therefore the following adaptor is synthesized,

```
5' GACCGACGTCtgcctcGTATACCGGACCGcatagctCC    3' olig#1
3'    GCTGCAGacggagCATATGGCCTGGCgtatcgaGGACT 5' olig#2
 AvaII|AatII|       |AccI|PsrII |       |Bsu36I
```

where the Ava II and Aat II sites share one GC base pair, and the Acc I and Psr II sites share a different CG pair. The two 38-base oligo-nts are synthesized by a standard procedure described elsewhere in the present invention; the oligo-nts are annealed to each other. The bases shown in lower case are spacers. In a later step, we will cut this adaptor with both Aat II and Acc I; for both enzymes to cut efficiently, there must be at least five bas between the sites. Similarly, we will begin the construction of the obd gene by inserting DNA at the Psr II and Bsu36 I sites; thus these sites are separated by seven bases to allow simultaneous cuts.

The annealed adaptor is ligated with RF M13mp18 that has been cut with both Ava II and Bsu36 I and purified by HPLC or polyacrylamide gel electrophoresis (PAGE). Cells are transformed with the ligated DNA. DNA from colonies selected on LB agar with ampicillin

is screened by restriction digestion. The desired construction can be cut with Rsr II or Acc I, but not by any of the enzymes listed in Table 18. Plasmid DNA from colonies that have the predicted restriction digestion is sequenced in the region of the insert to verify the construction. This construction retains both the Ava II and the Bsu36 I sites. The resulting construct is called pLG1.

The plasmid pLG1 is grown by standard techniques and DNA isolated and cut with both Aat II and Acc I. After ligation, there will still be Aat II and Acc I restriction sites at the ends of the inserted DNA. The Aat II-to-Acc I fragment of pBR322 is ligated to the backbone of LG1. The ligated DNA is used to transform competent E. coli that are plated on ampicillin-containing plates after a short grow-out.

Ampicillin-resistant colonies are picked. Plasmid DNA of the phagemid from the resistant colonies are digested with Bsu36 I and Rsr I. To verify the construction, DNA from phagemids with the correct restriction digestion pattern is sequenced: a) from about 20 bases above the Bsu36 I site to about 20 bases below the Rsr I site, and b) for about 30 bases either side of the unique Ava II site. The correct construct is named pLG2.

The Acc I restriction site is no longer needed for vector construction. To eliminate this site, RF pLG2 dsDNA is cut with Acc I, treated with Klenow fragment and dATP and dTTP to make it blunt and then religated. The ligated DNA is used to transform competent cells; after a short grow-out, ampicillin-resistant colonies are selected. Restriction digestion is used to screen

phagemid DNA from these colonies; the desired product cannot be cut with Acc I. To verify the construction, DNA from colonies lacking an Acc I restriction site is sequenced from about 20 bases above the former Acc I site to about 20 bases below it. The cloning vector, named pLG3, is now ready for stepwise insertion of the osp-ipbd gene.

We are now ready to design a gene (See Sec. 4) that will cause BPTI-domains to appear on the outer surface of an M13 derivative: LG7.

To obtain a novel protein domain attached to the outside of M13, we insert DNA that codes for mature BPTI after A23 of the precoat protein of M13. Mature BPTI begins with an arginine residue, which is charged; cleavage by signal peptidase I is normal in such cases. Signal peptidase I (SP-I) cuts a chimera of M13 coat protein and BPTI after A23 leaving mature BPTI attached at its carboxy end to the amino terminus of M13 CP.

The following amino-acid sequence, called AA_seq2, is constructed, by inserting the sequence for mature BPTI (shown underscored) immediately after the signal sequence of M13 precoat protein (indicated by the arrow) and before the sequence for the M13 CP.

AA_seq2

```
          1    1    2    12   3    3    4    4    5
     5    0    5    0    ⇓5   0    5    0    5    0
 5   MKKSLVLKASVAVATLVPMLSFA RPDFCLEPPYTGPCKARIIRYFYNAKA

          5    6    6    7    7    8    8    9    9   10
     5    0    5    0    5    0    5    0    5    0
10   GLCQTFVYGGCRAKRNNFKSAEDC RTCGGAAEGDDPAKAAFNSLQASAT

         10   11   11   12   12   13
     5    0    5    0    5    0
15   EYIGYAWAMVVVIVGATIGIKLFKKFTSKAS
```

We adopt the convention that sequence numbers of fusion proteins refer to the fusion, as coded, unless otherwise noted. Thus the alanine that begins M13 CP is referred to as "number 82", "number 1 of M13 CP", or "number 59 of the mature BPTI-M13 CP fusion".

The osp-ibbd gene is regulated by the lacUV5 promoter, so that the level of expression can be regulated by the concentration of IPTG supplied in the growth medium. (See Sec. 4.1). The host strain of E. coli should harbor the $lacI^q$ gene that represses the lacUV5 promoter to a greater extent than $lacI^+$. The osp-ibbd gene is ended by the trp attenuator so that RNA polymerase will not read through into subsequent genes. The osp-ibbd gene is expressed and processed in parallel with the wild-type gene VIII. The novel protein, that consists of BPTI tethered to a M13 CP domain, constitutes only a fraction of the coat. Affinity separation is able to separate phage carrying only five or six copies of a molecule that has high affinity for an affinity matrix (SMITH85); if incorporation of the chimeric protein results in about 30 copies of the protein exposed on the surface. If

this is insufficient, additional copies may be provided.

Figure 9 shows, in stereo, a hypothetical model of a short segment of the coat of a derivative of M13 in which some coat protein monomers are fusions of mature BPTI to the amino terminus of the normal M13 CP. The figure shows only protein C_{alpha}s: the DNA, not shown, lies inside the cylinder. The model of M13 coat is after the model for f1 of Marvin and colleagues (BANN81). The BPTI domain is taken from the Brookhaven Protein Data Bank entry "6PTI" and was attached by standard model building methods that insure that covalent bond lengths and angles are close to acceptable values. The space between the alpha helical main chains is filled by protein side groups so that the DNA is protected from solvent. The figure is not meant to suggest that BPTI fused to M13 CP will adopt the conformation shown, which is arbitrary. Rather the model shows that the fusion protein could fit into the supramolecular structure in a stereochemically acceptable fashion without disturbing the internal structure of either the M13 CP or BPTI domain.

The osp-inhd gene will use: a) the lacUV5 promoter, b) a Shine-Dalgarno sequence having high homology to natural Shine-Dalgarno sequences, c) a completely synthetic coding region having codons assigned to optimize placement of restriction sites, and d) the trp attenuator as transcriptional terminator. (See Secs. 4.1 and 4.2).

The ambiguous DNA sequence coding for AA_seq2, shown in Table 3, is examined by PROSPECT for places where recognition sites for any of the enzymes listed

in Table 21 could be created without altering the amino-acid sequence. (See Sec. 4.3). A master table of enzymes is compiled from the catalogues of enzyme suppliers listed in Table 4. The enzymes listed in Table 21 are those that do not cut the OCV, the construction of which is described above. The codes used in the ambiguous DNA are shown in Table 1.

Using the procedure given in Sec. 4.3, we design a ipbd gene, such as that shown in Table 22 and in Table 23. The recognition sequences of commercially available enzymes that recognize five or more bases are shown in Table 4. Some of these enzymes (e.g. Ban I or Hph I) cut the OCV too often to be of value. A summary of restriction sites in the designed pbd gene are given in Table 24.

The entire DNA sequence of the m13cp-ipbd fusion with annotation appears in Table 25 showing the useful restriction sites and biologically important features, viz. the lacUV5 promoter, the lacO operator, the Shine-Dalgarno sequence, the amino acid sequence, the stop codons, and the transcriptional terminator.

The ipbd gene is synthesized in several steps using the method described in Sec. 5.1, generating ds DNA fragments of 150 to 190 base pairs. In this example, the 3' overlap window (N_w) is set to run from 23 to 27 which is generous. The end spacers (N_s) that are added to insure efficient digestion are set to 8, which is also generous. Syntheses designed with smaller overlaps and shorter spacers would allow longer fragments of dsDNA to be synthesized and consume less of the reagents. Note, however, that Oliphant and Struhl (OLIP87) required large excesses of restriction

enzymes meant to cut near the ends of their dsDNA; this could have been because they had set N_S=2.

All DNA synthesis and purification is done by standard methods as described in Sec. 5.2.

The four steps (See Sec. 6.1) by which we clone synthetic fragments of the pljcp-btti gene (the osp-inbd gene of the present example) into pLGJ and its derivatives are illustrated in Figure 20.

The sequence to be introduced into pLGJ is shown in Table 26 and in Table 27. The segment is 158 bases long and is synthesized from two shorter synthetic oligo-nts as described in Sec. 5.1 of the generic specification. The important features of this segment are five restriction sites, the lacUV5 promoter, a Shine-Dalgarno site, and the TrpA attenuator as shown in Table 26.

Table 27 repeats the anti-sense strand shown in Table 26. The 99 base fragment shown in upper case letters and underscored (5'-CCGTCC....CCTTCG-3' = olig=3) is synthesized in the standard manner. Similarly, the 100 base long fragment of the sense strand shown in lower case (5'-cgctca....aattg-3' = olig=4) is synthesized. After annealing, the double-stranded region is extended with Klenow fragment by the procedure given above to make the entire 176 bases double stranded. The overlap region is 23 base pairs long and contains 14 CG pairs and 9 AT pairs. The DNA between Avr II and Apa II does not code for anything in the final pbd gene; it is there so that the DNA can be cut by both Avr II and Apa II at the same time in the next step. This spacer was made rich in C and G so

that annealing of the two single-stranded DNA fragments will be efficient. Eight bases have been added to the left of Rsr II and nine bases have been added to the left of Sau I (same specificity and cutting pattern as BsuJ6 I). These bases at the ends are not part of the final product; they must be present so that the restriction enzymes can bind and cut the synthetic DNA to produce specific sticky ends.

The synthetic DNA is cut with both Sau I and Rsr II and purified by HPLC or PAGE. RF pLG3 is cut with Sau I and Ava II and purified by HPLC or agarose gel electrophoresis and electroelution. The large piece from the phagemid and the synthetic DNA are ligated and used to transform E. coli. Ampicillin-resistant colonies are obtained and plasmids are screened by endonuclease digestion of RF phagemid DNA. The desired product can be cut by Avr II, Asu II, or BstE II, but the original phagemid can not be cut by any of these enzymes. To verify the insert; DNA from isolates that have the correct restriction sites is sequenced from about 10 bases above the Sau I site to about 10 bases below the Rsr II site. The construct with the correct insert is called pLG4.

The second step of the construction of the OCV is illustrated in Tables 28 and 29. This second segment of DNA is 155 bases long. As in the construction of pLG4, two pieces of single-stranded DNA are synthesized. A 99 base long fragment of the anti-sense strand (5'-GCACCA....CGTCCG-3' = olig=5) is shown in upper case letters and underscored; the other piece of 99 bases (5'-gatcta.....atcacct-3' = olig=6) is shown in lower case and is a fragment of the sense strand. These strands are complementary over 24 bases,

containing 14 CG base pairs and 10 AT base pairs. Klenow fragment is used to extend in both directions to produce ds DNA. Both the synthetic dsDNA and RF pLG4 DNA are cut with both Avr II and Asu II and purified by HPLC or the appropriate type of gel electrophoresis. The backbone from the phagemid pLG4 and the synthetic DNA are ligated and used to transform E. coli. Ampicillin-resistant colonies are obtained and plasmids are screened by restriction digestion. The desired product can be cut by any of Afl II, Nhe I, Nru I, Kpn I, Acc III, Ava I, Xho I, PflM I, Apa I, Dra II, Pss I, or BssH I while pLG4 can not be cut by any of these enzymes. To verify the insert, DNA from phagemids with the correct restriction sites is sequenced from about 10 bases above the BstE II site to about 10 bases below the Avr II site. The construct carrying this second insert is called pLG5.

Construction of pLG6 proceeds similarly to the construction of pLG5. The sequences are shown in Tables 30 and 31. The two single stranded segments (olig=7 and olig=8) are synthesized, annealed, and extended with Klenow fragment. The overlap region comprises 25 base pairs, 15 CG and 10 AT. Both the synthetic DNA and RF pLG5 are cut with both BssH I and Asu II, purified, and the appropriate pieces are ligated and used to transform E. coli. Ampicillin-resistant colonies are obtained and plasmids are screened by restriction digestion. The desired phagemid can be cut with any of Stu I, Acc I, Xca I, Esp I, Xca III, Srh I, Bbe I, or Nar I, while pLG5 can not be cut by any of these enzymes. To verify the third insert, DNA from phagemids with the correct restriction map is sequenced from about 10 bases above Asu II site to about 10 bases below the BssH I site.

The construct with the correct third insert is called pLG6.

The construction of pLG7 is illustrated in Tables 32 and 33 and proceeds similarly to the constructions of pLG4, pLG5, and pLG6. The two single stranded segments (olig#9 and olig#10) are synthesized, annealed, and extended with Klenow fragment. Both the synthetic DNA and RF pLG6 are cut with both Bbe I and Asu II, purified, and the appropriate pieces are ligated and used to transform E. coli. Ampicillin-resistant colonies are screened by restriction digestion of phagemid RF DNA. The desired phagemid can be cut with any of Sfi I, Hind III, Mlu I, BstX I, or Nco I, while pLG6 can be cut by none of these enzymes. To verify the fourth insert, DNA from phagemids with the correct restriction sites is sequenced from about 10 bases above the Asu II site to about 10 bases below the Bbe I site. The construct with the correct fourth insert is called pLG7; the display of BPTI on the outer surface of LG7 is verified by the methods of Sec. 8.

M13am429 is an amber mutation of M13 used to reduce non-specific binding by the affinity matrix for phages derived from M13. M13am429 is derived by standard genetic methods (MILL72) from wtM13. M13am429 is grown on E. coli strain PE384(F^+, SupE, Rec^-, Amp^S) and harvested by the standard method.

Phage LG7 is grown on E. coli strain PE384 in LB broth with various concentrations of IPTG added to the medium to induce the ompA-pbbd gene. Phage LG7 is obtained from cells grown with 0.0, 0.1, 1.0, 10.0 or 100.0 uM, or 1.0 mM IPTG, harvested (See Sec. 7) by the method of Salivar (SALI64), and concentrated to obtain

a titre of 10^{12} pfu/ml by the method of Messing (MESS83).

The preferred method of determining whether LG7 displays BPTI on its surface (See Sec. 8) is to determine whether these phage can retain a labeled derivative of trypsin (trp) or anhydrotrypsin (AHTrp) on a filter that allows passage of unbound trp or AHTrp. Trypsin contains 10 tyrosine residues and can be iodinated with ^{125}I by standard methods; we denote the labeled trypsin as "trp*". Labeled anhydrotrypsin is denoted as "AHTrp*". Other types of labels can be used on trp or AHTrp, e.g. biotin or a fluorescent label. AHTrp* or trp* is labeled to an activity of 0.1 uCi/ug. A sample of 10^{12} LG7(10 mM IPTG) is mixed with 1.0 ug of trp* or AHTrp* in 1.0 ml of a buffer of 10 mM KCl, adjusted to pH 8.0 with 1 mM K_2HPO_4 / KH_2PO_4. The mixture is passed through an Amicon MSP1 system fitted with a membrane filter that allows passage of proteins smaller that M_r = 300,000. Filters are soaked in buffer containing trp or AHTrp prior to the analysis. The filter is washed twice with 0.5 ml of buffer containing trp or AHTrp. The radioactivity retained on the filter is quantitated with a scintillation counter or other suitable device. If each virion displays one copy of BPTI, then .05 ug of protein can be bound that would give rise to 3×10^4 disintegrations / minute on the filter.

An alternative way to quantitate display of BPTI on the surface of LG7 is to use the stoichiometric binding between trypsin and BPTI to titrate the BPTI. A solution that titers 10^{12} pfu/ml of a phage is approximately 1.6×10^{-8} M in phage if each virion is infective. The ratio of pfu to total phage can be

determined spectrophotometrically using the molar extinction coefficients at 260 nm and 280 nm corrected for the increased length of LG7 as compared to wtM13. For example, if a 1.0 ml solution that contains 10^{12} pfu of LG7 phage grown with 1.0 mM IPTG inhibits trypsin solutions up to 4.8×10^{-7} M, we calculate that there are approximately 30 BPTIs/GP (i.e. (4.8×10^{-7} molecules of BPTI/l)/(1.6×10^{-8} phage/l)). Inhibition of a specified concentration of trypsin is most easily measured spectrophotometrically using a peptide-linked dye, such as N_{alpha}-benzoyl-Arg-Nan (TSCH 7).

Alternatively, binding to an affinity column may be used to demonstrate the presence of BPTI on the surface of phage LG7. An affinity column of 2.0 ml total volume having BioRad Affi-Gel 10(TM) matrix and 30 mg of AHTrp as affinity material is prepared by the method of BioRad. The void volume (V_V) of this column is, by hypothesis, 1.0 ml. This affinity column is denoted (AHTrp).

A sample of 10^{12} M13am429 is applied to (AHTrp) in 1.0 ml of 10 mM KCl buffered to pH 8.0 with KH_2PO_4 / K_2HPO_4. The column is then washed with the same buffer until the optical density at 280 nm of the effluent returns to base line or 4 x V_V have been passed through the column, whichever comes first. Samples of LG7 or LG10 are then applied to the blocked (AHTrp) column at 10^{12} pfu/ml in 1.0 ml of the same buffer. The column is then washed again with the same buffer until the optical density at 280 nm of the effluent returns to base line or 4 x V_V have been passed through, whichever comes first. Following this wash, a gradient of KCl from 10 mM to 2 M in 3 x V_V, buffered to pH 8.0 with phosphate is passed over the column. The first KCl

gradient is followed by a KCl gradient running from 2 M to 5 M in 3 x V_V. The second KCl gradient is followed by a gradient of guanidinium Cl from 0.0 M to 2.0 M in 2 x V_V in 5 M KCl and buffered to pH 8.0 with phosphate. Fractions of 50 ul are collected and assayed for phage by plating 4 ul of each fraction at suitable dilutions on sensitive cells. Retention of phage on the column is indicated by appearance of LG7 phage in fractions that elute significantly later from the column than control phage LG10 or wtM13. A successful isolate of LG7 that displays BPTI is identified, the bpti insert and junctions are sequenced, and this isolate is used for further work described below. It is likely that a significant fraction of clonal isolates from the same ligation that are characterized an identical by restriction digestion will similarly display BPTI.

If vgDNA is used to obtain a functional fusion between a BPTI mutant and M13 CP (vide infra), then DNA from a clonal isolate is sequenced in the regions that were variegated. Then gratuitous restriction sites for useful restriction enzymes are removed if possible by silent codon changes as follows. A de novo piece of synthetic DNA is synthesized such that the selected amino acid sequence is preserved and cloned into pLG7. The sequence numbers of residues in OSP-IPBD will be changed by any insertions; hereinafter, we will, however, denote residues inserted after residue 23 as 23a, 23b, etc. Insertions after residue 81 will be denoted as 81a, 81b, etc. This preserves the numbering of residues between C5 in BPTI and C55 in BPTI. Residue C5 of BPTI is always denoted as 28 in the fusion; residue C55 of BPTI is always denoted as 78 in the fusion, and the intervening residues have constant

numbers.

Should LG7 phage from cells grown with 10 mM IPTG fail to display BPTI on its surface, we have several options. We might try to determine why the construction failed to work as expected. There are various possible modes of failure, including : a) BPTI is not cleaved from the M13 signal sequence, b) BPTI is cleaved from the M13 CP, and c) the chimeric protein is made and cleaved after the signal sequence, but the processed protein is not incorporated into the M13 coat. BPTI has been secreted from E. coli (MARK86); however the M13 coat-protein signal sequence was not used. Therefore problems stemming from the signal sequence are unlikely, but possible. We could determine whether BPTI was present in the periplasm or bound to the inner membrane of LG7-infected cells by assays using labeled trypsin or anhydrotrypsin.

Proteins in the periplasm can be freed through spheroplast formation using lysozyme and EDTA in a concentrated sucrose solution (BIRD67, MALN94). If BPTI were free in the periplasm, it would be found in the supernatant. Trypsin labeled with ^{125}I would be mixed with supernatant and passed over a non-denaturing molecular sizing column and the radioactive fractions collected. The radioactive fractions would then be analyzed by SDS-PAGE and examined for BPTI-sized bands by silver staining.

Spheroplast formation exposes proteins anchored in the inner membrane. Spheroplasts would be mixed with AHTrp* and then either filtered or centrifuged to separate them from unbound AHTrp*. After washing with hypertonic buffer, the spheroplasts would be analyzed

for extent of AHTrp* binding.

If BPTI were found free in the periplasm, then we would expect that the chimeric protein was being cleaved both between BPTI and the M13 mature coat sequence and between BPTI and the signal sequence. In that case, we should alter the BPTI/M13 CP junction by inserting vgDNA at codons for residues 78-82 of AA_seq2.

If BPTI were found attached to the inner membrane, then two hypotheses can be formed. The first is that the chimeric protein is being cut after the signal sequence, but is not being incorporated into LG7 virion; the treatment would also be to insert vgDNA between residues 78 and 82 of AA_seq2. The alternative hypothesis is that BPTI could fold and react with trypsin even if signal sequence is not cleaved. N-terminal amino acid sequencing of trypsin-binding material isolated from cell homogenate determines what processing is occurring. If signal sequence were being cleaved, we would use the procedure above to vary residues between C78 and A82; subsequent passes would add residues after residue 81. If signal sequence were not being cleaved, we would vary residues between 23 and 27 of AA_seq2. Subsequent passes through that process would add residues after 23.

If BPTI were found neither in the periplasm nor on the inner membrane, then we would expect that the fault was in the signal sequence or the signal-sequence-to-BPTI junction. The treatment in this case would be to vary residues between 23 and 27.

Analytical experiments to determine what has gone

wrong take time and effort and, for the foreseen outcomes, indicate variations in only two regions. Therefore, we believe it prudent to try the synthetic experiments described below without doing the analysis. For example, these six experiments that introduce variegation into the bpti-gene VIII fusion could be trie

1) 3 variegated codons between residues 78 and 82 using olig#12 and olig#13,

2) 3 variegated codons between residues 23 and 27 using olig#14 and olig#15,

3) 5 variegated codons between residues 78 and 82 using olig#13 and olig#12a,

4) 5 variegated codons between residues 23 and 27 using olig#15 and olig#14a,

5) 7 variegated codons between residues 78 and 82 using olig#13 and olig#12b, and

6) 7 variegated codons between residues 23 and 27 using olig#15 and olig#14b.

To alter the BPTI-M13 CP junction, we introduce DNA variegated at codons for residues between 78 and 82 into the Sph I and Sfi I sites of pLG7. The residues after the last cysteine are highly variable in amino acid sequences homologous to BPTI, both in composition and length; in Table 25 these residues are denoted as G79, G80, and A81. The first part of the M13 CP is denoted as A82, E83, and G84. One of the oligo-nts olig#12, olig#12a, or olig#12b and the primer olig#13

are synthesized by standard methods. The oligo-nts are:

```
     residue   75  76  77  78  79  80  81  82  83
5' gc|gag|cGC|ATG|CGT|ACC|TGC|qfk|qfk|qfk|GCT|GAA|-

       84  85  86  87  88  89  90  91
     GGT|GAT|GAT|CCG|GCC|AAA|GCG|GCC|gcg|cc 3' olig=12

     residue   75  76  77  78  79  80  81 81a 81b
5' gc|gag|cGC|ATG|CGT|ACC|TGC|qfk|qfk|qfk|qfk|qfk|-

           82  83  84  85  86  87
         GCT|GAA|GGT|GAT|GAT|CCG|-

                     88  89  90  91
                   GCC|AAA|GCG|GCC|gcg|cc 3' olig=12a

     residue   75  76  77  78  79  80  81 81a 81b
5' gc|gag|cGC|ATG|CGT|ACC|TGC|qfk|qfk|qfk|qfk|qfk|-

           81c 81d 82  83  84  85  86  87
           qfk|qfk|GCT|GAA|GGT|GAT|GAT|CCG|-

                     88  89  90  91
                   GCC|AAA|GCG|GCC|gcg|cc 3' olig=12b


 residue   91  90  89  88  87  86
5' gg|cgc|GGC|CGC|TTT|GGC|CGG|ATC 3'   olig=13
```

where q is a mixture of (0.26 T, 0.18C, 0.26 A, and 0.30 G), f is a mixture of (0.22 T, 0.16 C, 0.40 A, and 0.22 G), and k is a mixture of equal parts of T and G. The bases shown in lower case at either end are spacers and are not incorporated into the cloned gene. The primer is complementary to the 3' end of each of the longer oligo-nts. One of the variegated oligo-nts and the primer olig=13 are combined in equimolar amounts and annealed. The dsDNA is completed with all four (nt)TPs and Klenow fragment. The resulting dsDNA and RF pLG7 are cut with both Sfi I and Sph I, purified,

mixed, and ligated. This ligation mixture goes through the process described in Sec. 15 in which we select a transformed clone that, when induced with IPTG, binds AHTrp.

To vary the junction between M13 signal sequence and BPTI, we introduce DNA variegated at codons for residues between 23 and 27 into the Kpn I and Xho I sites of pLG7. The first three residues are highly variable in amino acid sequences homologous to BPTI. Homologous sequences also vary in length at the amino terminus. One of the oligo-nts olig=14, olig=14a, or olig=14b and the primer olig=15 are synthesized by standard methods. The oligo-nts are:

```
 residue :      17  18  19  20  21  22  23  24  25
5'  g|gcc|gcG|GTA|CCG|ATG|CTG|TCT|TTT|GCT|qfk|qfk|-

      26  27  28  29  30
 |qfk|TTC|TGT|CTC|GAG|cgc|ccg|cga  3' olig=14


  residue     17  18  19  20  21  22  23  24  25  26
5'g|gcc|gcG|GTA|CCG|ATG|CTG|TCT|TTT|GCT|qfk|qfk|qfk|-

  26a 26b  27  28  29  30
 |qfk|qfk|TTC|TGT|CTC|GAG|cgc|ccg|cga|  3' olig=14a,


  residue     17  18  19  20  21  22  23  24  25  26
5'g|gcc|gcG|GTA|CCG|ATG|CTG|TCT|TTT|GCT|qfk|qfk|qfk|-

  26a 26b 26c 26d  27  28  29  30
 |qfk|qfk|qfk|qfk|TTC|TGT|CTC|GAG|cgc|ccg|cga|3'olig=14b


5'   |tcg|cgg|gcg|CTC|GAG|ACA|GAA|  3'  olig=15
```

where q is a mixture of (0.26 T, 0.18 C, 0.26 A, and 0.30 G), f is a mixture of (0.22 T, 0.16 C, 0.40 A, and

0.22 G), and K is a mixture of equal parts of T and G. The bases shown in lower case at either end are spacers and are not incorporated into the cloned gene. One of the variegated oligo-nts and the primer are combined in equimolar amounts and annealed. The ds DNA is completed with all four (nt)TPs and Klenow fragment. The resulting dsDNA and RF pLG7 are cut with both Kpn I and Xho I, purified, mixed, and ligated. This ligation mixture goes through the process described in Sec. 15 in which we select a transformed clone that, when induced with IPTG, binds AHTrp or trp.

Other numbers of variegated codons could be used.

If none of these approaches produces a working chimeric protein, we may try a different signal sequence. If that doesn't work, we may try a different OSP in M13 because the structural data clearly indicate that BPTI could not be joined to the carboxy terminus. The next best OSP of M13 is the gene III protein because there is fusion data (SMIT85, CRU288).

Example 1, Part II

BPTI binds very tightly to trypsin (K_d = 6.0 x 10^{-14} M) and to anhydrotrypsin, so that these molecules are not preferred for optimizing the amount of BPTI to display on LG7 or the amount of affinity molecule to attach to the column. Tschesche et al. reported on the binding of several BPTI derivatives to various proteases:

Dissociation constants for BPTI derivatives, Molar.

Residue #15	Trypsin (bovine pancreas)	Chymotrypsin (bovine pancreas)	Elastase (porcine pancreas)	Elastase (human leukocytes)
lysine	6.0×10^{-14}	9.0×10^{-9}	-	3.5×10^{-6}
glycine	-	-	+	7.0×10^{-9}
alanine	+	-	2.8×10^{-8}	2.5×10^{-9}
valine	-	-	5.7×10^{-8}	1.1×10^{-10}
leucine	-	-	1.9×10^{-8}	2.9×10^{-9}

From the report of Tschesche et al. we infer that molecular pairs marked "+" have K_ds greater than 3.5×10^{-6} M and that molecular pairs marked "-" have K_ds much greater than 3.5×10^{-6} M. Because of the wealth of data about the binding of BPTI and various mutants to trypsin and other proteases (TSCH87), we can proceed in various ways. (For other PBDs we can obtain two different monoclonal antibodies, one with a high affinity having K_d of order 10^{-11} M, and one with a moderate affinity having K_d on the order of 10^{-9} M.) In this example, we may use: a) the moderate binding between BPTI and human leukocyte elastase (HuLE1), b) the moderately strong binding of porcine elastase to BPTI(V15), or c) the binding of BPTI(A15) (residue 15 in the pbd gene) for trypsin (weak but detectable) or for porcine pancreatic elastase.

Following the teachings of Sec. 10, we compare the retention of LG7 virions to the retention of wild-type M13 on (AHTrp). M13 derivatives having more DNA than wild-type M13 have corresponding longer virions. Thus we will create pLG5 that differs from pLG7 only in having stop codons at codons 2 and 3, and an altered L

codon at codon 7 of the csp-ipbd gene. Phage LG8 will have exactly as much DNA as LG7; therefore the LG8 virion is exactly as long as the LG7 virion. LG8 can not, however, display BPTI on its surface. To generate these mutations we synthesize the oligo-nt

5' (121)|aac|gct|agc|ctt|Cag|aac|cag|aga|ttA|ctA|cat|-
|agt|gaq|cct| (80) 3' olig=11

that is complementary to bases 80 through 121 of the ipbd gene, shown in Table 23, except for the three upper-case, underscored bases. Olig=11 and the primers olig=24, olig=25, and olig=26 are annealed to circular ssDNA from LG7. Klenow fragment (from US Biochemical) and all four (nt)TPs are used to complete the circular dsDNA. After treatment with Klenow fragment, the dsDNA is treated with ligase. Cells are transformed with the ligated dsDNA and, after a short grow-out, the cells are plated on ampicillin-containing LB agar. By changing the third base in codon 7, we have destroyed the unique Afl II site in pLG7. Thus we can screen colonies for loss of the Afl II site. To confirm the construction, DNA from plaques with no Afl II site are sequenced from about base 140 to about base 40 of the csp-ipbd gene.

To expedite identification of different M13-derived phage, we replace the amp^R gene of LG3 with the tet^R gene from pBR322. Plasmid pBR322 is cut with Bsm I at the unique site at 1353 and the linearized DNA is blunted with Klenow fragment and purified. The blunt DNA is cut with Aat II and the 1428-base tet^R-bearing fragment purified by agarose gel electrophoresis or HPLC. Plasmid pLG8 ds DNA is cut with Xba I at the unique site and the linearized DNA is

blunted with Klenow fragment and purified. The linear, blunt DNA is digested with Ant II and the 7.8 Kb fragment is isolated. The two isolated DNA fragments are mixed, annealed, ligated, and used to transform competent E. coli cells. The transformed cells are selected with tetracycline. The correct construction contains Sal I, EcoR I, and EcoR V sites, but LG8 contains none of these. The correct construction, having 9.2 kb, is easily distinguished from pBR322 and is called LG10. DNA from phage LG10 is sequenced in the vicinity of the junctions of the newly inserted tet^R gene to confirm the construction.

The phage LG7 is grown at various levels of IPTG in the medium and harvested in the way previously described. An affinity column having bed volume of 2.0 ml and supporting an amount of HuLE1 picked from the range 0.1 mg to 30.0 mg on 1 ml of BioRad Affi-Gel 10(TM) or Affi-Gel 15(TM) is designated (HuLE1). An appropriate set of densities of HuLE1 on the column is (0.1 mg/ml, 0.5 mg/ml, 2.0 mg/ml, 8.0 mg/ml, 15.0 mg/ml, and 30.0 mg/ml). The V_V of (HuLE1) is, by hypothesis, 1.0 ml. The elution of LG7 phage is compared to the elution of LG10 on (HuLE1) having varying amounts of HuLE1 affixed. The columns are eluted in a standard way:

1) 10 mM KCl buffered to pH 8.0 with phosphate, until optical density at 280nm falls to base line or 4 x V_V, whichever is first,

2) a gradient of 10 mM to 2 M KCl in 3 x V_V, pH held at 8.0 with phosphate,

3) a gradient of 2 M to 5 M KCl in 3 x V_V,

phosphate buffer to pH 8.0,

4) constant 5 M KCl plus 0 to 0.8 M guanidinium Cl in 2 x V_V, with phosphate buffer to pH 8.0.

The preferred level of induction ($IPTG_{optimal}$) and amount of affinity molecule on the matrix ($DoAMoM_{optimal}$) are those settings that give the sharpest LG7 elution peak that shows significant retardation as compared to LG8, which carries no BPTI. By hypothesis, the best separation occurs for the amount of BPTI/GP produced when the cells are induced with 10.0 uM IPTG and when 4.0 mg HuLE1/ml is applied to BioRad Affi-Gel 10(TM).

When the amount of BPTI/GP and the amount of HuLE1/volume of support have been optimized, we turn to optimization of elution rate, initial ionic strength, and the amount of GP/(volume of support). These parameters can be optimized separately.

Using optimal BPTI/GP and HuLE1/volume of support, we measure the elution volume of LG7 and LG8 for different elution rates, viz. 1, 1/2, 1/4, 1/8 and 1/16 times the maximum flow rate. M13 is shear resistant, so that the pressure that can be applied across the column is limited only by the mechanical properties of the support material. By hypothesis, 1/4 of maximum elution rate is better than 1/2, but 1/8 is about the same as 1/4. Therefore 1/4 maximum elution rate will be used.

Elution volumes of LG7 obtained from cells grown on media that is 2.0 mM in IPTG are measured at optimal DoAMoM and elution rate for loadings of 10^9, 10^{10},

10^{11}, and 10^{12} pfu. By hypothesis, 10^{12} pfu of pure LG7 overloads the column and significant number of phage elute before their characteristic position in the KCl gradient. We also find that 10^{11} pfu overloads the column only slightly, and that 1010 pfu does not overload the column. Because the use of the affinity separation in Sec. 15 will involve a population in which no single member is more than one part in 10^4, we conclude that 10^{12} pfu of a variegated population could be applied to a column of 1.0 ml matrix volume without overloading with respect any one species. The overloading of a 1.0 ml column by 10^{12} pfu also indicates that the initial column that captures indiscriminately adhesive phage should be 5 to 10 times as large as the column that supports the target material.

Elution volumes of LG7 and LG10 obtained from cells grown on media that is 2.0 mM in IPTG are measured at optimal DoAMoM and elution rate and for a loading of 10^{10} pfu for various initial ionic strengths: 1.0 mM, 5.0 mM, 10.0 mM, 20.0 mM, and 50.0 mM. We find that LG10 is slightly retarded by the column when loaded at 1.0 mM KCl, but that LG7 always comes off the column at its characteristic place in the gradient. We use 10.0 mM as initial ionic strength in all remaining affinity separations.

To determine the sensitivity of chromatography of phage that display variants of BPTI on their surfaces (Sec. 10.1), we prepare artificial mixtures of two closely-related phage that differ only at one residue in the BPTI domain. One variety of phage has strong affinity for the column used in this step, while the other phage has no affinity for the column. We

chromatograph these mixtures to discover how little of the phage that binds to the column can be detected within a large majority of phage that do not bind the column.

For these tests we choose AHTrp as AfM(BPTI). A column having 2 ml bed volume is prepared with (DoAMoM$_{optimal}$ mg of AHTrp)/(ml of Affi-Gel 10$^{(TM)}$). The column is called (AHTrp) and has V_V = 1.0 ml.

A new phage, LG9, is prepared that displays BPTI(V15) as IPBD in contrast to LG7 that displays BPTI(K15, wild-type) as IPBD. Residue 15 of BPTI is residue 38 of the osp-ipbd gene. We introduce the change K38 to V by replacement of a short segment of the osp-ipbd gene. The two oligo-nts

	g	p	c	V	a	r	i	i	r	y	f	
	35	36	37	38	39	40	41	42	43	44	45	
5′		C	TGt	gtt	GCt	CGt	ATa	ATa	CGc	TAT	TTC	-
3′	cc	ggg	acA	CAA	cgA	gcA	taT	taT	gcG	ata	aag	-

Apa I (BssH II)

	y	n	a	k	a	g	
	46	47	48	49	50	51	
	TAC	AAC	GCT	AAA	GCA	GG	3′ olig=16
	atg	ttg	cga	ttt	cgt	cc	5′ olig=17

Stu I

are synthesized by standard methods and annealed; the lower case letters in olig=16 and the upper case letters in olig=17 are mutant with respect to pLG7. Plasmid pLG7 DNA is digested with both Apa I and Stu I and the large piece purified. The ds oligo-nt is added to the purified backbone of pLG7 and ligated; the ligated DNA is used to transform competent cells. After a short grow out, the cells are plated on

ampicillin-containing plates and Amp^R colonies are picked. The mutations destroy the unique BssH II site, thus we can screen colonies through restriction digestion. To confirm the construction, DNA from colonies having the correct restriction digestion pattern is sequenced from about 10 bases above the Stu I site to about 10 bases below the Apa I site. The correct construction is called pLG9.

To expedite differentiation between LG7 and an LG9-derivative phage, we replace the amp^R gene of LG9 with the tet^R gene from pBR322. Plasmid pBR322 is cut with Bsa I at the unique site at 1353 and the linearized DNA is blunted with Klenow fragment and purified. The blunt DNA is cut with Aat II and the 1420-base tet^R-bearing fragment purified by agarose gel electrophoresis or HPLC. Plasmid pLG9 ds DNA is cut with Xba I at the unique site and the linearized DNA is blunted with Klenow fragment and purified. The linear, blunt DNA is digested with Aat II and the 7.8 kb fragment is isolated. The two isolated DNA fragments are mixed, annealed, ligated, and used to transform competent E. coli cells. The transformed cells are selected with tetracycline. The correct construction contains Sal I, EcoR I, and EcoR V sites, but LG9 contains none of these. The correct construction, having 9.2 kb, is easily distinguished from pBR322 and is called LG11. DNA from phage LG11 is sequenced in the vicinity the junctions of the newly inserted tet^R gene to confirm the construction.

LG7 and LG11 are grown with optimum IPTG (2.0 mM) and harvested. Mixtures are prepared in the ratios

$$LG7:LG11 :: 1:V_{lim}$$

where V_{lim} ranges from 10^{10} to 10^5 by factors of 10. Large values of V_{lim} are tested first; once a V_{lim} is found that allows recovery of LG7, smaller values of V_{lim} are not be tested. Once a value of V_{lim} is found that allows recovery of LG7, we test values that are larger by 2-, 4-, or 8-fold so that V_{lim} is determined within a factor of 2.

The column (AHTrp) is first blocked by treatment with 10^{11} virions of M13am429 in 100 ul of 10 mM KCl buffered to pH 8.0 with phosphate; the column is washed with the same buffer until OD_{260} returns to base line or 4 x V_V have passed through the column, whichever comes first. One of the mixtures of LG7 and LG11 containing 10^{12} pfu in 1 ml of the same buffer is applied to (AHTrp). The column is eluted in a standard way :

1) 10 mM KCl buffered to pH 8.0 with phosphate, until optical density at 280nm falls to base line or 4 x V_V, whichever is first, (discard effluent),

2) a gradient of 10 mM to 2 M KCl in 3 x V_V, pH held at 8.0 with phosphate, (30 x 100 ul fractions),

3) a gradient of 2 M to 5 M KCl in 3 x V_V, phosphate buffer to pH 8.0, (30 x 100 ul fractions),

4) constant 5 M KCl plus 0 to 0.8 M guanidinium Cl in 2 x V_V, with phosphate buffer to pH 8.0, (20 x 100 ul fractions),

5) constant 5 M KCl plus 0.8 M guanidinium Cl in 1.2 x V_V, with phosphate buffer to pH 8.0, (12 x 100 ul fractions).

Samples of 4 ul from each fraction are plated at suitable dilution on phage-sensitive Sup^+ cells (so that M13am429 will not grow). In addition to the effluent fractions, a sample is removed from the column and used as an inoculum for phage-sensitive Sup^+ cells. Plaques are transferred to ampicillin-containing LB agar. Colonies that are ampicillin-resistant are tested for display of BPTI(K15) by use of trp^+ or $\Delta HTrp^+$. Testing begins with colonies obtained by culturing an inoculum from the column, proceeds to the last effluent fraction, and works backwards toward earlier fractions. Once a positive colony is found, no further tests are required for that value of V_{lim}. If no BPTI positive colonies are detected, the population of phage obtained from the column matrix and the last few (e.g. 5 to 10) phage-bearing fractions are merged and cultured. Phage are harvested from this culture and chromatographed by the above procedure. This process continues until a positive colony is isolated or N_{chrom} passes of chromatography and growth have been completed. If no positive colonies are detected after N_{chrom} passes of enrichment, Vlim is reduced by a suitable factor and the process is repeated.

By hypothesis, $V_{lim} = 4.0 \times 10^8$ is the largest value for which LG7 can be recovered. Thus $C_{sensi} = 4.0 \times 10^3$. Three cycles of chromatography are required to isolate LG7, so the first approximation to C_{eff} is 740 (= $\exp(\ln_e(4.0 \times 10^8)/3)$).

We now determine the efficiency of the affinity separation (Sec. 10.2). This is done by: a) preparing mixtures of LG7 and LG11 in the ratio 1:Q, b) enriching the population for LG7 for one separation cycle, and c) determining the fraction of LG7 in the last phage-bearing fraction. The phage are obtained from cultures induced at 10.0 uM IPTG, the optimal level. Q is decreased until roughly half the phage are LP7. We start with Q = 1.5×10^4 = 20 x approximate C_{eff}. The mixture is applied to a (AHTrp) column bearing 4.0 mg AHTrp on 1.0 ml of Affi-Gel 10 (the optimal DoAMoM) and eluted in the specified manner. A sample of 4 ul from each fraction is plated at suitable dilution on phage sensitive cells on LB agar. The identity of colonies in the last phage-bearing fraction is determined by transferring colonies to ampicillin-containing and tetracycline-containing plates; colonies that show Tet^R are from LG11 and colonies that show Amp^R are from LG7. When Q is 1.5×10^4, 5% of colonies are BPTI positive. When Q is 1.5×10^3, 60% of the colonies are BPTI positive. Thus we calculate C_{eff} = .60 x 1.5×10^3 = 900.

Myoglobin is strongly colored and it is possible that binding of HHMb to M13 could provide enough optical absorption to allow FACS sorting of M13 that bind HHMb (See Sec. 10.4).

We have now constructed LG7 that displays one or more BPTI domains on each virion. The osp-inbd gene is under control of the lacUV5 promoter so that expression levels of BPTI-M13 CP can be manipulated via [IPTG]. This construct may be used to develop many different binding proteins, all based on BPTI. An optimum level of induction has been determined. An optimum amount of

$AfM(PBD) = DoAMoM_{optimum}$ = 2.0 mg/(ml of support) has been determined; target molecules will be applied to columns at this level in the process disclosed in Sec. 15.1. These optimum levels may be adequate for all targets and all variegations of BPTI displayed on derivatives of M13 based on LG7, but some further optimization may be needed if other values of pH or temperatures are used.

Other pbd gene fragments may be substituted for the bpti gene fragment in pLG7 with a high likelihood that PBD will appear on the surface of the new LG7 derivative.

Example 1, Part III

HHMb is chosen as a typical protein target; any other protein could be used. HHMb satisfies all of the criteria for a target: 1) it is large enough to be applied to an affinity matrix, 2) after attachment it is not reactive, and 3) after attachment there is sufficient unaltered surface to allow specific binding by PBDs.

The essential information for HHMb is known: 1) HHMb is stable at least up to 70°C, between pH 4.4 and 9.3, 2) HHMb is stable up to 1.6 M Guanidinium Cl, 3) the pI of HHMb is 7.0, 4) for HHMb, M_r = 16,000, 5) HHMb requires haem, 6) HHMb has no proteolytic activity.

In addition, the following information about HHMb and other myoglobins is available: 1) the sequence of HHMb is known, 2) the 3D structure of sperm whale myoglobin is known; HHMb has 19 amino acid differences

and it is generally assumed that the 3D structures are almost identical, 3) HHMb has no enzymatic activity, 4) HHMb is not toxic.

We set the specifications of an SBD as :

1) T = 25°C

2) pH = 8.0

3) Acceptable solutes :
   A) for binding :
      i) phosphate, as buffer, 0 to 20 mM, and
      ii) KCl, 10 mM,
   B) for column elution :
      i) phosphate, as buffer, 0 to 30 mM,
      ii) KCl, up to 5 M, and
      iii) Guanidinium Cl, up to 0.8 M.

4) Acceptable $K_d < 1.0 \times 10^{-8}$ M.

We choose LG7 as GP(IPBD).

As stated in Sec. 13.1, the residues to be varied are picked, in part, through the use of interactive computer graphics to visualize the structures. In this section, all residue numbers refer to BPTI. We pick a set of residues that forms a surface such that all residues can contact one target molecule. Information that we refer to during the process of choosing residues to vary includes: 1) the 3D structure of BPTI, 2) solvent accessibility of each residue as computed by the method of Lee and Richards (LEEB71), 3) a compilation of sequences of other proteins homologous to BPTI, and 4) knowledge of the structural nature of

different amino acid types.

Tables 16 and 34 indicate which residues of BPTI: a) have substantial surface exposure, and b) are known to tolerate other amino acids in other closely related proteins. We use interactive computer graphics to pick sets of eight to twenty residues that are exposed and variable and such that all members of one set can touch a molecule of the target material at one time. If BPTI has a small amino acid at a given residue, that amino acid may not be able to contact the target simultaneously with all the other residues in the interaction set, but a larger amino acid might well make contact. A charged amino acid might affect binding without making direct contact. In such cases, the residue should be included in the interaction set, with a notation that larger residues might be useful. In a similar way, large amino acids near the geometric center of the interaction set may prevent residues on either side of the large central residue from making simultaneous contact. If a small amino acid, however, were substituted for the large amino acid, then the surface would become flatter and residues on either side could make simultaneous contact. Such a residue should be included in the interaction set with a notation that small amino acids may be useful.

Table 35 was prepared from standard model parts and shows the maximum span between C_{beta} and the tip of each type of side group. C_{beta} is used because it is rigidly attached to the protein main-chain; rotation about the C_{alpha}-C_{beta} bond is the most important degree of freedom for determining the location of the side group.

Table 33 indicates five surfaces that meet the given criteria. The first surface comprises the set of residues that actually contacts trypsin in the complex of trypsin with BPTI as reported in the Brookhaven Protein Data Bank entry "1TPA". This set is indicated by the number "1". The exposed surface of the residues in this set (taken from Table 16) totals 1148 A^2. Although this is not strictly the area of contact between BPTI and trypsin, it is approximately the same.

Other surfaces, numbered 2 to 5, were picked by first picking one exposed, variable residue and then picking neighboring residues until a surface was defined. The choice of sets of residues shown in Table 34 is in no way exhaustive or unique; other sets of variable, surface residues can be picked. Set #2 is shown in stereo view, Figure 10, including the alpha carbons of BPTI, the disulfide linkages, and the side groups of the set. We take the orientation of BPTI in Figure 10 as a standard orientation and hereinafter refer to K15 as being at the top of the molecule, while the carboxy and amino termini are at the bottom.

Solvent accessibilities are useful, easily tabulated indicators of a residue's exposure. Solvent accessibilities must be used with some caution: small amino acids are under-represented and large amino acids over-represented. The user must consider what the solvent accessibility of a different amino acid would be when substituted into the structure of BPTI.

To create specific binding between a derivative of BPTI and HRMb, we will vary the residues in set #2. This set includes the twelve principal residues 17(R), 19(I), 21(Y), 27(A), 28(G), 29(L), 31(Q), 32(E), 34(V),

48(A), 49(E), and 52(M) (Sec. 13.1.1). None of the residues in set #2 is completely conserved in the sample of sequences reported in Table 34; thus we can vary them with a high probability of retaining the underlying structure. Independent substitution at each of these twelve residues of the amino acid types observed at that residue would produce approximately 4.4×10^9 amino acid sequences and the same number of surfaces.

BPTI is a very basic protein. This property has been used in isolating and purifying BPTI and its homologues so that the high frequency of arginine and lysine residues may reflect bias in isolation and is not necessarily required by the structure. Indeed, SCI-III from Bombyx mori contains seven more acidic than basic groups (SASA84).

Residue 17 is highly variable and fully exposed and can contain R, K, A, Y, H, F, L, M, T, G, Y, P, or S. All types of amino acids are seen: large, small, charged, neutral, and hydrophobic. That no acidic groups are observed may be due to bias in the sample.

Residue 19 is also variable and fully exposed, containing P, R, I, S, K, Q, and L.

Residue 21 is not very variable, containing F or Y in 31 of 33 cases and I and W in the remaining cases. The side group of Y21 fills the space between T32 and the main chain of residues 47 and 48. The OH at the tip of the Y side group projects into the solvent. Clearly one can vary the surface by substituting Y or F so that the surface is either hydrophobic or hydrophilic in that region. It is also possible that

the other aromatic amino acid (viz. H) or the other hydrophobics (L, M, or V) might be tolerated.

Residue 27 most often contains A, but S, K, L, and T are also observed. On structural grounds, this residue will probably tolerate any hydrophilic amino acid and perhaps any amino acid.

Residue 28 is G in BPTI. This residue is in a turn, but is not in a conformation peculiar to glycine. Six other types of amino acids have been observed at this residue: K, N, Q, R, H, and M. Small side groups at this residue might not contact HHMb simultaneously with residues 17 and 34. Large side groups could interact with HHMb at the same time as residues 17 and 34. Charged side groups at this residue could affect binding of HHMb on the surface defined by the other residues of the principal set. Any amino acid, except perhaps P, should be tolerated.

Residue 29 is highly variable, most often containing L. This fully exposed position will probably tolerate almost any amino acid except, perhaps, P.

Residues 31, 32, and 34 are highly variable, exposed, and in extended conformations; any amino acid should be tolerated.

Residues 48 and 49 are also highly variable and fully exposed, any amino acid should be tolerated.

Residue 52 is in an alpha helix. Any amino acid, except perhaps P, might be tolerated.

Now we consider possible variation of the secondary set (Sec. 13.1.2) of residues that are in the neighborhood of the principal set. Neighboring residues that might be varied at later stages include 9(P), 11(T), 15(K), 16(A), 18(I), 20(R), 22(F), 24(N), 26(K), 35(Y), 47(S), 50(D), and 53(R).

Residue 9 is highly variable, extended, and exposed. Residue 9 and residues 48 and 49 are separated by a bulge caused by the ascending chain from residue 31 to 34. For residue 9 and residues 48 and 49 to contribute simultaneously to binding, either the target must have a groove into which the chain from 31 to 34 can fit, or all three residues (9, 48, and 49) must have large amino acids that effectively reduce the radius of curvature of the BPTI derivative.

Residue 11 is highly variable, extended, and exposed. Residue 11, like residue 9, is slightly far from the surface defined by the principal residues and will contribute to binding in the same circumstances.

Residue 15 is highly varied. The side group of residue 15 points away form the face defined by set #2. Changes of charge at residue 15 could affect binding on the surface defined by residue set #2.

Residue 16 is varied but points away from the surface defined by the principal set. Changes in charge at this residue could affect binding on the face defined by set #2.

Residue 18 is I in BPTI. This residue is in an extended conformation and is exposed. Five other amino acids have been observed at this residue: M, F, L, V,

and T. Only T is hydrophilic. The side group points directly away from the surface defined by residue set #2. Substitution of charged amino acids at this residue could affect binding at surface defined by residue set #2.

Residue 20 is R in BPTI. This residue is in an extended conformation and is exposed. Four other amino acids have been observed at this residue: A, S, L, and Q. The side group points directly away from the surface defined by residue set #2. Alteration of the charge at this residue could affect binding at surface defined by residue set #2.

Residue 22 is only slightly varied, being Y, F, or H in 30 of 33 cases. Nevertheless, A, N, and S have been observed at this residue. Amino acids such as L, M, I, or Q could be tried here. Alterations at residue 22 may affect the mobility of residue 21; changes in charge at residue 22 could affect binding at the surface defined by residue set #2.

Residue 24 shows some variation, but probably can not interact with one molecule of the target simultaneously with all the residues in the principal set. Variation in charge at this residue might have an effect on binding at the surface defined by the principal set.

Residue 26 is highly varied and exposed. Changes in charge may affect binding at the surface defined by residue set #2; substitutions may affect the mobility of residue 27 that is in the principal set.

Residue 35 is most often Y, W has been observed.

The side group of 35 is buried, but substitution of F or W could affect the mobility of residue 34.

Residue 47 is always T or S in the sequence sample used. The O_{gamma} probably accepts a hydrogen bond from the NH of residue 50 in the alpha helix. Nevertheless, there is no overwhelming steric reason to preclude other amino acid types at this residue. In particular, other amino acids the side groups of which can accept hydrogen bonds, viz. N, D, Q, and E, may be acceptable here.

Residue 50 is often an acidic amino acid, but other amino acids are possible.

Residue 53 is often R, but other amino acids have been observed at this residue. Changes of charge may affect binding to the amino acids in interaction set #2.

Stereo Figure 10 shows the residues in set #2, plus R39. From Figure 10, one can see that R39 is on the opposite side of BPTI form the surface defined by the residues in set #2. Therefore, variation at residue 39 at the same time as variation of some residues in set #2 is much less likely to improve binding that occurs along surface #2 than is variation of the other residues in set #2.

In addition to the twelve principal residues and 13 secondary residues, there are two other residues, 30(C) and 33(F), involved in surface #2 that we will probably not vary, at least not until late in the procedure. These residues have their side groups buried inside BPTI and are conserved. Changing these

residues does not change the surface nearly so much as does changing residues in the principal set. These buried, conserved residues do, however, contribute to the surface area of surface #2. The surface of residue set #2 is comparable to the area of the trypsin-binding surface. Principal residues 17, 19, 21, 27, 28, 29, 31, 32, 34, 48, 49, and 52 have a combined solvent-accessible area of 946.9 A^2. Secondary residues 9, 11, 15, 16, 18, 20, 22, 24, 26, 35, 47, 50, and 53 have combined surface of 1041.7 A^2. Residues 30 and 33 have exposed surface totaling 38.2 A^2. Thus the three groups' combined surface is 2026.8 A^2.

Residue 30 is C in BPTI and is conserved in all homologous sequences. It should be noted, however, that C14/C38 is conserved in all natural sequences, yet Marks et al. (MARK87) showed that changing both C14 and C38 to A,A or T,T yields a functional trypsin inhibitor. Thus it is possible that BPTI-like molecules will fold if C30 is replaced.

Residue 33 is F in BPTI and in all homologous sequences. Visual inspection of the BPTI structure suggests that substitution of Y, M, H, or L might be tolerated.

Having identified twenty residues that define a possible binding surface, we must choose some to vary first. Given our hypothetical affinity separation sensitivity, C_{sensi}, we decide to vary six residues leaving some margin for errors in the actual base composition of variegated bases. To obtain maximal recognition, we choose residues from the principal set that are as far apart as possible. Table 16 shows the distances between the beta carbons of residues in the

principal and peripheral set. R17 and V34 are at one end of the principal surface. Residues A27, G28, L29, A48, E49, and M52 are at the other end, about twenty Angstroms away; of these, we will vary residues 17, 27, 29, 34, and 48. Residues 28, 49, and 52 will be varied at later rounds.

Of the remaining principal residues, 21 is left to later variations. Among residues 19, 31, and 32, we arbitrarily pick 19 to vary.

Unlimited variation of six residues produces 6.4×10^7 amino acid sequences. By hypothesis, C_{sensi} is 1 in 4×10^8. Table 37 shows the programmed variegation at the chosen residues. The parental sequence is present as 1 part in 5.5×10^7, but the least favored sequences are present at only 1 part in 4.2×10^9. Among single-amino-acid substitutions from the PPBD, the least favored is F17-I19-A27-L29-V34-A48 and has a calculated abundance of 1 part in 1.6×10^8. Using the optimal qfk codon, we can recover the parental sequence and all one-amino-acid substitutions to the PPBD if actual nt compositions come within 5% of programmed compositions. The number of transformants is $M_{ntv} = 1.0 \times 10^9$ (also by hypothesis), thus we will produce most of the programmed sequences.

The residue numbers of the preceding section are referred to mature BPTI (R1-P2-...-A58). Table 25 has residue numbers referring to the pre-MIJCP-BPTI protein: all mature BPTI sequence numbers have been increased by the length of the signal sequence, i.e. 23. Thus in terms of the pre-OSP-PBD residue numbers, we wish to vary residues 40, 42, 50, 52, 57, and 71. A DNA subsequence containing all these codons is found

between the (Apa I/Dra II/Pss I) sites at base 191 and the Sph I site at base 309 of the osp-pbd gene. Among Apa I, Dra I, and Pss I, Apa I is preferred because it recognizes six bases without any ambiguity. Dra II and Pss I, on the other hand, recognize six bases with two-fold ambiguity at two of the bases. The vgDNA will contain more Dra II and Pss I recognition sites at the varied locations than it will contain Apa I recognition sites. The unwanted extraneous cutting of the vgDNA by Apa I and Sph I will eliminate a few sequences from our population. This is a minor problem, but by using the more specific enzyme (Apa I), we minimize the unwanted effects. The sequence shown in Table 37 illustrates an additional way in which gratuitous restriction sites can be avoided in some cases. The osp-lpbd gene had the codon GGC for g51; because we are varying both residue 50 and 52, it is possible to obtain an Apa I site. If we change the glycine codon to GGT, the Apa I site can no longer arise. Apa I recognizes the DNA sequence (GGGCC/C).

Each piece of dsDNA to be synthesized needs six to eight bases added at either end to allow cutting with restriction enzymes and is shown in Table 37. The first synthetic base (before cutting with Apa I and Sph I) is 184 and the last is 322. There are 142 bases to be synthesized. The center of the piece to the synthesized lies between Q54 and V57. The overlap can not include varied bases, so we choose bases 245 to 256 as the overlap that is 12 bases long. Note that the codon for F56 has been changed to TTC to increase the GC content of the overlap. The amino acids that are being varied are marked as X with a plus over them. Codons 57 and 71 are synthesized on the sense (bottom) strand. The design calls for "gfk" in the antisense

strand, so that the sense strand contains (from 5' to 3') a) equal part C and A (i.e. the complement of k), b) (0.40 T, 0.22 A, 0.22 C, and 0.16 G) (i.e. the complement of f), and c) (0.26 T, 0.26 A, 0.30 C, and 0.18 G).

Each residue that is encoded by "qfk" has 21 possible outcomes, each of the amino acids plus stop. Table 12 gives the distribution of amino acids encoded by "qfk", assuming 5% errors. The abundance of the parental sequence is the product of the abundances of R x I x A x L x V x A. The abundance of the least-favored sequence is 1 in 4.2×10^9.

Olig#27 and olig#28 are annealed and extended with Klenow fragment and all four (nt)TPs. Both the ds synthetic DNA and RF pLG7 DNA are cut with both Apa I and Sph I. The cut DNA is purified and the appropriate pieces ligated (See Sec. 14.1) and used to transform competent PE383. (Sec. 14.2). In order to generate a sufficient number of transformants, V_C is set to 5000 ml.

1) culture E. coli in 5.0 l of LB broth at 37°C until cell density reaches 5×10^7 to 7×10^7 cells/ml,

2) chill on ice for 65 minutes, centrifuge the cell suspension at 4000g for 5 minutes at 4°C,

3) discard supernatant; resuspend the cells in 1667 ml of an ice-cold, sterile solution of 60 mM $CaCl_2$,

4) chill on ice for 15 minutes, and then

centrifuge at 4000g for 5 minutes at 4°C,

5) discard supernatant; resuspend cells in 2 x 400 ml of ice-cold, sterile 60 mM $CaCl_2$; store cells at 4°C for 24 hours,

6) add DNA in ligation or TE buffer; mix and store on ice for 30 minutes; 20 ml of solution containing 5 ug/ml of DNA is used,

7) heat shock cells at 42°C for 90 seconds,

8) add 200 ml LB broth and incubate at 37°C for 1 hour,

9) add the culture to 2.0 l of LB broth containing ampicillin at 35-100 ug/ml and culture for 2 hours at 37°C,

10) centrifuge at 8000 g for 20 minutes at 4°C,

11) discard supernatant, resuspend cells in 50 ml of LB broth plus ampicillin and incubate 1 hour at 37°C,

12) plate cells on LB agar containing ampicillin,

13) harvest virions by method of Salivar et al. (SALI64).

The heat shock of step (7) can be done by dividing the 200 ml into 100 200 ul aliquots in 1.5 ml plastic Eppendorf tubes. It is possible to optimize the heat

shock for other volumes and kinds of container. It is important to: a) use all or nearly all the vgDNA synthesized in ligation, this will require large amounts of pLG7 backbone, b) use all or nearly all the ligation mixture to transform cells, and c) culture all or nearly all the transformants at high density. These measures are directed at maintaining diversity.

IPTG is added to the growth medium at 2.0 mM (the optimal level) and virions are harvested in the usual way (Sec. 14.3). It is important to collect virions in a way that samples all or nearly all the transformants. Because F⁻ cells are used in the transformation, multiple infections do not pose a problem.

HHMb has a pI of 7.0 and we carry out chromatography at pH 8.0 so that HHMb is slightly negative while BPTI and most of its mutants are positive. HHMb is fixed (Sec. 15.1) to a 2.0 ml column on Affi-Gel 10(TM) or Affi-Gel 15(TM) at 4.0 mg/ml support matrix, the same density that is optimal for a column supporting trp.

We note that charge repulsion between BPTI and HHMb should not be a serious problem and does not impose any constraints on ions or solutes allowed as eluants. Neither BPTI nor HHMb have special requirements that constrain choice of eluants. The eluant of choice is KCl in varying concentrations.

To remove variants of BPTI with strong, indiscriminate binding for any protein or for the support matrix (Sec. 15.2), we pass the variegated population of virions over a column that supports bovine serum albumin (BSA) before loading the

population onto the (HHMb) column. Affi-Gel 10(TM) or Affi-Gel 15(TM) is used to immobilize BSA at the highest level the matrix will support. A 10.0 ml column is loaded with 5.0 ml of Affi-Gel-linked-BSA; this column, called (BSA), has V_V = 5.0 ml. The variegated population of virions containing 10^{12} pfu in 1 ml (0.2 x V_V) of 10 mM KCl, 1 mM phosphate, pH 8.0 buffer is applied to (BSA). We wash (BSA) with 4.5 ml (0.9 x V_V) of 50 mM KCl, 1 mM phosphate, pH 8.0 buffer. The wash with 50 mM salt will elute virions that adhere slightly to BSA but not virions with strong binding. The pooled effluent of the (BSA) column is 5.5 ml of approximately 13 mM KCl.

The column (HHMb) is first blocked by treatment with 10^{11} virions of M13(am429) in 100 ul of 10 mM KCl buffered to pH 8.0 with phosphate; the column is washed with the same buffer until OD_{260} returns to base line or 2 x V_V have passed through the column, whichever comes first. The pooled effluent from (BSA) is added to (HHMb) in 5.5 ml of 13 mM KCl, 1 mM phosphate, pH 8.0 buffer. The column is eluted (Sec. 15.3) in the following way:

1) 10 mM KCl buffered to pH 8.0 with phosphate, until optical density at 280nm falls to base line or 2 x V_V, whichever is first, (effluent discarded),

2) a gradient of 10 mM to 2 M KCl in 3 x V_V, pH held at 8.0 with phosphate, (30 x 100 ul fractions),

3) a gradient of 2 M to 5 M KCl in 3 x V_V, phosphate buffer to pH 8.0 (30 x 100 ul

fractions),

4) constant 5 M KCl plus 0 to 0.8 M guanidinium Cl in 2 x V_V, with phosphate buffer to pH 8.0, (20 x 100 ul fractions), and

5) constant 5 M KCl plus 0.8 M guanidinium Cl in 1 x V_V, with phosphate buffer to pH 8.0, (10 x 100 ul fractions).

In addition to the elution fractions, a sample is removed from the column and used as an inoculum for phage-sensitive Sup^- cells (Sec. 15.4). A sample of 4 ul from each fraction is plated on phage-sensitive Sup^- cells. Fractions that yield too many colonies to count are replated at lower dilution. An approximate titre of each fraction is calculated. Starting with the last fraction and working toward the first fraction that was titered, we pool fractions until approximately 10^9 phage are in the pool, i.e. about 1 part in 1000 of the phage applied to the column. This population is infected into 3×10^{11} phage-sensitive PE304 in 300 ml of LB broth. The very low multiplicity of infection (moi) is chosen to reduce the possibility of multiple infection. After thirty minutes, viable phage have entered recipient cells but have not yet begun to produce new phage. Phage-born genes are expressed at this phase, and we can add ampicillin that will kill uninfected cells. These cells still carry F-pili and will absorb phage helping to prevent multiple infections.

If multiple infection should pose a problem that cannot be solved by growth at low multiple-of-infection on F^- cells, the following procedure can be employed to

obviate the problem. Virions obtained from the affinity separation are infected into F^+ E. coli and cultured to amplify the genetic messages (Sec. 15.5). CCC DNA is obtained either by harvesting RF DNA or by in vitro extension of primers annealed to ss phage DNA. The CCC DNA is used to transform F^- cells at a high ratio of cells to DNA. Individual virions obtained in this way should bear only proteins encoded by the DNA within.

The variegation produced as many as 6.4×10^7 different amino-acid sequences. C_{eff} is 900. Thus, after two separation cycles, the probability of isolating a single SBD is less than 0.10; after three cycles, the probability rises above 0.10.

The phagemid population is grown and chromatographed three times and then examined for SBDs (Sec. 15.7). In each separation cycle, phage from the last three fractions that contain viable phage are pooled with phage obtained by removing some of the support matrix as an inoculum. At each cycle, about 10^{12} phage are loaded onto the column and about 10^9 phage are cultured for the next separation cycle. After the third separation cycle, 32 colonies are picked from the last fraction that contained viable phage; phage from these colonies are denoted SBD1, SBD2,..., and SBD32.

Each of the SBDs is cultured and tested for retention on a Pep-Tie column supporting HHMb (Sec. 15.8). Phage LG7(SBD11) shows the greatest retention on the Pep-Tie (HHMb) column, eluting at 367 mM KCl while wtM13 elutes at 20 mM KCl. SBD11 becomes the parental amino-acid sequence to the second variegation

cycle.

The result of this hypothetical experiment is shown in Table 38. R40 changed to D, I42 changed to Q, A50 changed to E, L52 remained L, and A71 changed to W.

The next round of variegation (Sec. 16) is illustrated in Table 39. The residues to be varied are chosen by: a) choosing some of the residues in the principal set that were not varied in the first round (viz. residues 42, 44, 51, 54, 55, 72, or 75 of the fusion), and b) choosing some residues in the secondary set. Residues 51, 54, 55, and 72 are varied through all twenty amino acids and, unavoidably, stop. Residue 44 is only varied between Y and F. Some residues in the secondary set are varied through a restricted range: primarily to allow different charges (+, 0, -) to appear. Residue 38 is varied through K, R, E, or G. Residue 41 is varied through I, V, K, or E. Residue 43 is varied through R, S, G, N, K, D, E, T, or A.

Olig#29 and olig#30 are synthesized, annealed, extended and cloned into pLG7 at the Apa I/Sph I sites. The ligation mixture is used to transform 5 l of competent PE383 cells so that 10^9 transformants are obtained. A new (HHMb) is constructed using the same support matrix as was used in round 1. A sample of 10^{12} of the harvested LG7 are applied to (HHMb) and affinity separated. The last 10^9 phage off the column and an inoculum are pooled and cultured. The cultured phagemids are re-chromatographed for three separation cycles. Thirty-two clonal isolates (denoted SBD11-1, SBD11-2,..., SBD11-32) are obtained from the effluent of the third separation cycle and tested for binding on

a Pep-Tie (HHMb) column. Of this set, SBD11-23 shows the greatest retention on the Pep-Tie (HHMb) column, eluting at 692 mM KCl.

The results of this hypothetical selection is shown in Table 40. Residue 38 (K15 of BPTI) changed to E, 41 becomes V, 42 goes to N, 44 goes to F, 51 goes to F, 54 goes to S, 55 goes to A, and 72 goes to Q.

The sbd11-23 portion of the osp-obd gene is cloned into and expression vector and BPTI(E15, D17, V18, Q19, N20, F21, E27, F28, L29, S31, A32, S34, W71, Q72) is expressed in the periplasm. This protein is isolated by standard methods and its binding to HHMb is tested. K_d is found to be 4.5×10^{-7} M.

A third round of variation, using SBD11-23 as PPBD, is illustrated in Table 41; eight amino acids are varied. Those in the principal set, residues 40, 55, and 57, are varied through all twenty amino acids. Residue 32 is varied through P, Q, T, K, A, or E. Residue 34 is varied through T, P, Q, K, A, or E. Residue 44 is varied through F, L, Y, C, W, or stop. Residue 50 is varied through E, K, or Q. Residue 52 is varied through L, F, I, M, or V.

The result of this variation is shown in Table 42. The selected SBD is denoted SBD11-23-5 and elutes from a Pep-Tie (HHMb) column at 980 mM KCl. The sbd11-23-5 segment is cloned into an expression vector and BPTI(E9, Q11, E15, A17, V18, Q19, N20, W21, Q27, F28, M29, S31, L32, H34, W71, Q72) is produced. This time the K_d is 7.3×10^{-9} M.

This example is hypothetical. It is anticipated

that more variegation cycles will be needed to achieve dissociation constants of 10^{-8} M. It is also possible that more than three separation cycles will be needed in some variegation cycles. Real DNA chemistry and DNA synthesizers may have larger errors than our hypothetical 5%. If $S_{err} > 0.05$, then we may not be able to vary six residues at once. Variation of 5 residues at once is certainly possible.

Table 1: Single-letter codes.

Single-letter code is used for proteins :

a = ALA	c = CYS	d = ASP	e = GLU	f = PHE
g = GLY	h = HIS	i = ILE	k = LYS	l = LEU
m = MET	n = ASN	p = PRO	q = GLN	r = ARG
s = SER	t = THR	v = VAL	w = TRP	y = TYR
. = STOP	* = any amino acid			

b = n or d
z = e or q
x = any amino acid

Single-letter IUB codes for DNA :

T, C, A, G stand for themselves

M for A or C
R for puRines A or G
W for A or T
S for C or G
Y for pYrimidines T or C
K for G or T

V for A, C, or G (not T)
H for A, C, or T (not G)
D for A, G, or T (not C)
B for C, G, or T (not A)

N for any base.

Table 2: Preferred Outer-Surface Proteins

Genetic Package	Preferred Outer-Surface Protein	Reason for preference
M13	coat protein (gpVIII)	a) exposed amino terminus, b) predictable post-translational processing, c) numerous copies in virion.
	gp III	a) fusion data available.
PhiX174	G protein	a) known to be on virion exterior, b) small enough that the G-inbd gene can replace H gene.
E. coli	LamB	a) fusion data available, b) non-essential.
B. subtilis spores	CotC	a) no post-translational processing, b) distinctive sdequence that causes protein to localize in spore coat, c) non-essential.
	CotD	Same as for CotC.

Table 3: Ambiguous DNA for AA_seq2

m 1 A.T.G	k 2 A.A.r	k 3 A.A.r	s 4 T.C.n A.G.y	l 5 T.T.r C.T.n	v 6 G.T.n	l 7 T.T.r C.T.n	k 8 A.A.r
a 9 G.C.n	s 10 T.C.n A.G.y	v 11 G.T.n	a 12 G.C.n	v 13 G.T.n	a 14 G.C.n	t 15 A.C.n	l 16 T.T.r C.T.n
v 17 G.T.n	p 18 C.C.n	m 19 A.T.G	l 20 T.T.r C.T.n	s 21 T.C.n A.G.y	f 22 T.T.y	a 23 G.C.n	r 24 C.G.n A.G.r
p 25 C.C.n	d 26 G.A.y	f 27 T.T.y	c 28 T.G.y	l 29 T.T.r C.T.n	e 30 G.A.r	p 31 C.C.n	p 32 C.C.n
y 33 T.A.y	t 34 A.C.n	g 35 G.G.n	p 36 C.C.n	c 37 T.G.y	k 38 A.A.r	a 39 G.C.n	r 40 C.G.n A.G.r
i 41 A.T.h	i 42 A.T.h	r 43 C.G.n	y 44 T.A.y	f 45 T.T.y	y 46 T.A.y	n 47 A.A.y	a 48 G.C.n
k 49 A.A.r	a 50 G.C.n	g 51 G.G.n	l 52 T.T.r C.T.n	c 53 T.G.y	q 54 C.A.r	t 55 A.C.n	f 56 T.T.y
v 57 G.T.n	y 58 T.A.y	g 59 G.G.n	g 60 G.G.n	c 61 T.G.y	r 62 C.G.n A.G.r	a 63 G.C.n	k 64 A.A.r

Table 3, continued.

r 65 C.G.n A.G.r	n 66 A.A.y	n 67 A.A.y	f 68 T.T.y	k 69 A.A.r	s 70 T.C.n A.G.y	a 71 G.C.n	e 72 G.A.r
d 73 G.A.y	c 74 T.G.y	m 75 A.T.G	r 76 C.G.n	t 77 A.C.n	c 78 T.G.y	q 79 G.G.n	g 80 G.G.n
a 81 G.C.n	a 82 G.C.n	e 83 G.A.r	g 84 G.G.n	d 85 G.A.y	d 86 G.A.y	p 87 C.C.n	a 88 G.C.n
k 89 A.A.r	a 90 G.C.n	a 91 G.C.n	f 92 T.T.y	N 93 A.A.y	s 94 T.C.n A.G.y	l 95 T.T.r C.T.n	q 96 C.A.r
a 97 G.C.n	s 98 T.C.n A.G.y	a 99 G.C.n	t 100 A.C.n	e 101 G.A.r	y 102 T.A.y	i 103 A.T.h	g 104 G.G.n
y 105 T.A.y	a 106 G.C.n	w 107 T.G.G	a 108 G.C.n	m 109 A.T.G	v 110 G.T.n	v 111 G.T.n	v 112 G.T.n
i 113 A.T.h	v 114 G.T.n	g 115 G.G.n	a 116 G.C.n	t 117 A.C.n	i 118 A.T.h	g 119 G.G.n	i 120 A.T.h
k 121 A.A.r	l 122 T.T.r C.T.n	f 123 T.T.y	k 124 A.A.r	k 125 A.A.r	f 126 T.T.y	t 127 A.C.n	s 128 T.C.n A.G.y
k 129 A.A.r	a 130 G.C.n	s 131 T.C.n A.G.y	. 132 T.A.r T.G.A	. 133 T.A.r T.G.A	. 134 T.A.r T.G.A		

Table 4: Table of Restriction Enzymes

Table of restriction enzymes with IUB codes.

Suppliers :

S=Sigma Chemical Co.
P.O.Box 14508
St. Louis, Mo. 63178

B=Bethesda Research Laboratories
P.O.Box 6009
Gaithersburg, Maryland, 20877

M=Boehringer Mannheim Biochemicals
7941 Castleway Drive
Indianapolis, Indiana, 46250

I=International Biochemicals, Inc.
P.O.Box 9558
New Haven, Connecticutt, 06535

N=New England BioLabs
32 Tozer Road
Beverly, Massachusetts, 01915

P=Promega
2800 S. Fish Hatchery Road
Madison, Wisconsin, 53711

T=Stratagene Cloning Systems
11099 North Torrey Pines Road
La Jolla, California, 92037

+ before enzyme name means that overhang can not be self-complementary.
% before enzyme name means that overhang may or may not be self-complementary.

Table 4, continued.

	Enzyme	Recognit.	Symm	cuts	Supply
	Aat II	GACGTC	P	5, 1	<S,M,I,N,T
5	%Acc I	GTMKAC	P	2, 4	<B,M,I,N,P,T
	Acc III	TCCGGA	P	1, 5	<T
	Acy I	GRCGYC	P	2, 4	<Aha II:N
	Afl II	CTTAAG	P	1, 5	<N
	%Afl III	ACRYCT	P	1, 5	<none
10	Aha III	TTTAAA	P	3, 3	<B,T &Dra I:M,I,N, P
	+AlwN I	CAGNNNCTG	P	6, 3	<N
	Apa I	GGGCCC	P	5, 1	<M,I,N,P,T
	ApaL I	GTGCAC	P	1, 5	<N,T
15	Ase I	ATTAAT	P	2, 4	<N
	Asp718	GGTACC	P	1, 5	<none
	Asu II	TTCGAA	P	2, 4	<P,N(BstB I)
	%Ava I	CYCGRG	P	1, 5	<S?,B,M,I,N,P,T; Apu I:T
20	Ava III	ATGCAT	P	5, 1	<T; Nsi I:M,N,P,T; EcoT22 I:T
	Avr II	CCTAGG	P	1, 5	<N
	Bal I	TGGCCA	P	3, 3	<S,B,I,N,T
	BamH I	GGATCC	P	1, 5	<S,B,M,I,N,P,T
25	%Ban I	GGYRCC	P	1, 5	<M,I,N,T
	Bbe I	GGCGCC	P	5, 1	<
	%Bbv I	GCAGC	nP	13,17	<I,N,T
	%Bbv II	GAAGAC	nP	8,12	<
	Bcl I	TGATCA	P	1, 5	<S,B,M,I,N,T
30	+Bgl I	GCCNNNNNGGC	P	7, 4	<S,B,I,N,P,T
	Bgl II	AGATCT	P	1, 5	<S,B,I,N,P,T
	+Bin I	GGATC	nP	9,10	<Alw I:N
	+Bsm I	GAATGCN	nP	7, 5	<N,T
	BspH I	TCATGA	P	1, 5	<N
35	%BspM I	ACCTGC	nP	10,14	<N
	BssH II	GCGCGC	P	1, 5	<N,T
	+BstE II	GGTNACC	P	1, 6	<S,B,M,N,T
	%BstX I	CCANNNNNNTGG	P	6, 6	<N,P,T
	Cfr I	YGGCCR	P	1, 5	<Eae I:N,T
40	Cla I	ATCGAT	P	2, 4	<S,B,M,N,T; &Ban III:I
	+Dra II	RGGNCCY	P	2, 5	<M,T ; Eco0109 I:N
	+Dra III	CACNNNGTG	P	6, 3	<M,N,T
	Eco47 II	AGCGCT	P	3, 3	<none
45	+EcoN I	CCTNNNNNAGG	P	5, 6	<N(soon)
	EcoR I	GAATTC	P	1, 5	<S,B,M,I,N,P,T
	EcoR V	GATATC	P	3, 3	<S,B,M,I,N,P,T
	+Esp I	GCTNAGC	P	2, 5	<T
	%Fok I	GGATG	nP	14,18	<M ,N,T
50	Gdi II	YGGCCG	nP	1, 5	<
	Hae I	WGGCCW	P	3, 3	<
	Hae II	RGCGCY	P	5, 1	<S,B,M,I,N,T

Table 4, continued.

	+Hga I	GACGC	nP	10,15	<N
	%HgiA I	GWGCWC	P	5, 1	<N
5	%HgiC I	GGYRCC	P	0, 6	<
	%HgiJ II	GRGCYC	P	5, 1	<Ban II:S,M,I,N,T
	Hind II	GTYRAC	P	3, 3	<M; &Hinc II:S,B,I ,N,P,T
	Hind III	AAGCTT	P	1, 5	<S,B,M,I,N,P,T
10	Hpa I	GTTAAC	P	3, 3	<S,B,M,I,N,P ,T
	+Hph I	GGTGA	nP	13,12	<N,T
	Kpn I	GGTACC	P	5, 1	<S,B,M,I,N,P,T ; Asp718:M
	+Mbo II	GAAGA	nP	13,12	<S,B,I,N
15	Mlu I	ACGCGT	P	1, 5	<M,N,P,T
	Mst I	TGCGCA	P	3, 3	<T; Fsp I:S,N
	Nae I	GCCGGC	P	3, 3	<M,N,T
	Nar I	GGCGCC	P	2, 4	<B,N,T
	Nco I	CCATGG	P	1, 5	<B,M,N,P,T
20	Nde I	CATATG	P	2, 4	<B,N,T
	Nhe I	GCTAGC	P	1, 5	<M,N,P,T
	Not I	GCGGCCGC	P	2, 6	<M,N,P,T
	Nru I	TCGCGA	P	3, 3	<B,M,N,T
	Nsp7524	RCATGY	P	5, 1	<
25	Nsp8 II	CMGCKG	P	3, 3	<
	+PflM I	CCANNNNNTGG	P	7, 4	<N
	+Ple I	GAGTCNNNNN	nP	9,10	<N
	PmaC I	CACGTG	P	3, 3	<none
	+PmuM I	RGGWCCY	P	2, 5	<N
30	+Pss I	RGGNCCY	P	5, 2	<I
	Pst I	CTGCAG	P	5, 1	<S,B,M,I,N,P,T
	Pvu I	CGATCG	P	4, 2	<S,B,N,B(Xor II),M ,P,T
	Pvu II	CAGCTG	P	3, 3	<S,B,M,I,N,P,T
35	+Rsr II	CGGWCCG	P	2, 5	<N,T
	Sac I	GAGCTC	P	5, 1	<B(Sst I),M,I,N,P, T
	Sac II	CCGCGG	P	4, 2	<B(Sst II),I,N,P,T
	Sal I	GTCGAC	P	1, 5	<B,M,I,N,P,T
40	+Sau I	CCTNAGG	P	2, 5	<M; Cvn I:B; Mst II :T; Bsu36 I:N; Acc I:T
	Sca I	AGTACT	P	3, 3	<M,N,P,T
	%SfaN I	GCATC	nP	10,14	<N
	+Sfi I	GGCCNNNNNGGCC	P	5, 5	<N,P,T
45	Sma I	CCCGGG	P	3, 3	<B,M,I,N,P,T
	SnaB I	TACGTA	P	3, 3	<M,N,T
	Spe I	ACTAGT	P	1, 5	<M,N,T
	Sph I	GCATGC	P	5, 1	<B,M,I,N,P,T
	Ssp I	AATATT	P	3, 3	<M,N,T
50	Stu I	AGGCCT	P	3, 3	<M,N,I(Aat I),P,T
	%Sty I	CCWWGG	P	1, 5	<N,P,T

Table 4, continued.

```
     %Taq II GACCGA       nP 17,15 <none
     %Taq II'CACCCA       nP 17,15 <none
     +Tth111I GACNNNGTC    P  4, 5 <I,N,T
     %Tth111II CAARCA     nP 16,14 <none
     Xba I   TCTAGA        P  1, 5 <B,M,I,N,P,T
     Xca I   GTATAC        P  3, 3 <N(soon)
     Xho I   CTCGAG        P  1, 5 <B,M,I,P,T; Ccr I:
                                      T ; PaeR7 I:N
     Xho II  RGATCY        P  1, 5 <M,T ;N(BstY I)
     Xma I   CCCGGG        P  1, 5 <I,N,P,T
     Xna III CGGCCG        P  1, 5 <B; Eag I:N;
                                      Eco52 I:T
     Xmn I   GAANNNNTTC    P  5, 5 <N,M(Asp700),T

       N_restrct =    100
```

Notes:

Symm: P for palindromic, nP for non-palindromic

cuts: first number indicates position of cut in top strand, 1 means after first base of recognition; second number indicates position of cut in lower strand, counting left-to-right.

Table 5: Potential sites in ipbd gene.

Summary of cuts.

Enz = %Acc I has 3 elective sites : 96 169 281
Enz = Afl II has 1 elective sites : 19
Enz = Apa I has 2 elective sites : 102 103
Enz = Asu II has 1 elective sites : 381
Enz = Ava III has 1 elective sites : 314
Enz = BspM II has 1 elective sites : 72
Enz = BssH II has 2 elective sites : 67 115
Enz = %BstX I has 1 elective sites : 323
Enz = +Dra II has 3 elective sites : 102 103 226
Enz = +EcoN I has 2 elective sites : 62 94
Enz = +Fsp I has 2 elective sites : 57 187
Enz = Hind III has 6 elective sites : 9 23 60 287 361 386
Enz = Kpn I has 1 elective sites : 48
Enz = Mlu I has 1 elective sites : 314
Enz = Nar I has 2 elective sites : 238 343
Enz = Nco I has 1 elective sites : 323
Enz = Nhe I has 3 elective sites : 25 289 388
Enz = Nru I has 2 elective sites : 38 65
Enz = +PflM I has 1 elective sites : 94
Enz = PmaC I has 1 elective sites : 228
Enz = +PpuM I has 2 elective sites : 102 226
Enz = +Rsr II has 1 elective sites : 102
Enz = +Sfi I has 2 elective sites : 24 261
Enz = Spe I has 3 elective sites : 12 45 379
Enz = Sph I has 1 elective sites : 221
Enz = Stu I has 5 elective sites : 23 70 150 287 386
Enz = %Sty I has 6 elective sites : 11 44 143 261 323 383
Enz = Xba I has 1 elective sites : 84
Enz = Xca I has 2 elective sites : 96 169
Enz = Xho I has 1 elective sites : 85
Enz = Xma III has 3 elective sites : 70 209 242

Enzymes not cutting ipbd.

Avr II	BamH I	Bcl I	BstE II
EcoR I	EcoR V	Hpa I	Not I
Sac I	Sal I	Sau I	Sma I
Xma I			

Table 6: Exposure of amino acid types in T4 lzm & HEWL.

HEADER HYDROLASE (O-GLYCOSYL) 18-AUG-86 2LZM
COMPND LYSOZYME (E.C.3.2.1.17)
AUTHOR L.H.WEAVER,B.W.MATTHEWS

Coordinates from Brookhaven Protein Data Bank: 1LYM.

Only Molecule A was considered.

HEADER HYDROLASE(O-GLYCOSYL) 29-JUL-82 1LYM
COMPND LYSOZYME (E.C.3.2.1.17)
AUTHOR J.HOGLE,S.T.RAO,M.SUNDARALINGAM

Solvent radius = 1.40 Atomic radii in Table 7.

Surface area measured in Angstroms^2.

Type	N	<area>	sigma	max	min	Max exposed(fraction)
ALA	27	211.0	1.47	214.3	207.1	85.1(0.40)
CYS	10	239.8	3.56	245.5	234.4	38.3(0.16)
ASP	17	271.1	5.36	281.4	262.5	127.1(0.47)
GLU	10	297.2	5.78	304.9	285.4	100.7(0.34)
PHE	8	316.6	5.92	325.4	307.5	99.8(0.32)
GLY	23	185.5	1.31	188.3	183.3	91.9(0.50)
HIS	2	297.7	3.23	301.0	294.5	32.9(0.11)
ILE	16	278.1	3.61	285.6	269.6	57.5(0.21)
LYS	19	309.2	5.38	321.9	300.1	147.1(0.48)
LEU	24	282.6	6.75	304.0	269.8	109.9(0.39)
MET	7	293.0	5.70	299.5	283.1	88.2(0.30)
ASN	26	273.0	5.75	285.1	262.6	143.4(0.53)
PRO	5	239.9	2.75	242.1	234.6	128.7(0.54)
GLN	8	299.5	4.75	305.8	291.5	145.9(0.49)
ARG	24	344.7	8.66	355.8	326.7	240.7(0.70)
SER	16	228.6	3.59	236.6	223.1	96.2(0.42)
THR	18	250.3	3.89	257.2	244.2	139.9(0.56)
VAL	15	254.3	4.05	261.8	245.7	111.1(0.44)
TRP	9	359.4	3.38	366.4	355.1	102.0(0.28)
TYR	9	335.8	4.97	342.0	325.0	72.6(0.22)

Table 7: Atomic radii
Angstroms

C_{alpha}	1.70
$O_{carbonyl}$	1.52
N_{amide}	1.55
Other atoms	1.80

Table 8

Fraction of DNA molecules having n non-parental bases when reagents that have fraction M of parental nt.

M	.9965	.97716	.92612	.8577	.79433	.63096
f0	.9000	.5000	.1000	.0100	.0010	.000001
f1	.09499	.35061	.2393	.04977	.00777	.0000175
f2	.00485	.1188	.2768	.1197	.0292	.000149
f3	.00016	.0259	.2061	.1854	.0705	.000812
f4	.000004	.00409	.1110	.2077	.1232	.003207
f8	0.	$2x10^{-7}$	.00096	.0336	.1182	.020165
f16	0.	0.	0.	$5x10^{-7}$	.00006	.027281
f23	0.	0.	0.	0.	0.	.0000089
most	0	0	2	5	7	12

"most" is the value of n having the highest probability.

Table 9: best vgCodon

```
     Program "Find Optimum vgCodon."
     INITIALIZE-MEMORY-OF-ABUNDANCES
     DO ( t1 = 0.21 to 0.31 in steps of 0.01 )
     . DO ( c1 = 0.13 to 0.23 in steps of 0.01 )
     . . DO ( a1 = 0.23 to 0.33 in steps of 0.01 )
Comment      calculate g1 from other concentrations
     . . . g1 = 1.0 - t1 - c1 - a1
     . . . IF( g1 .ge. 0.15 )
     . . . . DO ( a2 = 0.37 to 0.50 in steps of 0.01 )
     . . . . . DO ( c2 = 0.12 to 0.20 in steps of 0.01 )
Comment      Force D+E = R + K
     . . . . . g2 = (g1*a2 -.5*a1*a2)/(c1+0.5*a1)
Comment      Calc t2 from other concentrations.
     . . . . . t2 = 1. - a2 - c2 - g2
     . . . . . IF(g2.gt. 0.1.and. t2.gt.0.1)
     . . . . . . CALCULATE-ABUNDANCES
     . . . . . . COMPARE-ABUNDANCES-TO-PREVIOUS-ONES
     . . . . . ..end_IF_block
     . . . . ..end_DO_loop ! c2
     . . . ..end_DO_loop ! a2
     . . ..end_IF_block ! if g1 big enough
     . ..end_DO_loop ! a1
     ..end_DO_loop ! c1
     ..end_DO_loop ! t1
     WRITE the best distribution and the abundances.
```

Table 10: Abundances obtained from optimum vgCodon

Amino acid	Abundance	Amino acid	Abundance
A	4.80%	C	2.86%
D	6.00%	E	6.00%
F	2.86%	G	6.60%
H	3.60%	I	2.86%
K	5.20%	L	6.82%
M	2.86%	N	5.20%
P	2.88%	Q	3.60%
R	6.82%	S	7.02% mfaa
T	4.16%	V	6.60%
W	2.86% lfaa	Y	5.20%
stop	5.20%		

ratio = Abun(W)/Abum(S) = 0.4074

i	(1/ratio)^j	(ratio)^j	stop-free
1	2.454	.4074	.9480
2	6.025	.1660	.8987
3	14.788	.0676	.8520
4	36.298	.0275	.8077
5	89.095	.0112	.7657
6	218.7	4.57×10^{-3}	.7258
7	536.8	1.86×10^{-3}	.6881

Table 11: Calculate worst codon.

```
      Program "Find worst vgCodon within Serr of given
 5            distribution."
      INITIALIZE-MEMORY-OF-ABUNDANCES
   Comment  Serr is % error level.
      READ  Serr
   Comment T1i,C1i,A1i,G1i, T2i,C2i,A2i,G2i, T3i,G3i
10 Comment are the intended nt-distribution.
      READ  T1i, C1i, A1i, G1i
      READ  T2i, C2i, A2i, G2i
      READ  T3i, G3i
      Fdwn = 1.-Serr
15    Fup  = 1.+Serr
      DO ( t1 = T1i*Fdwn to T1i*Fup in 7 steps)
      . DO ( c1 = C1i*Fdwn to C1i*Fup in 7 steps)
      . . DO ( a1 = A1i*Fdwn to A1i*Fup in 7 steps)
      . . . g1 = 1. - t1 - c1 - a1
20    . . . IF( (g1-G1i)/G1i .lt. -Serr)
   Comment  g1 too far below G1i, push it back
      . . . . g1 = G1i*Fdwn
      . . . . factor = (1.-g1)/(t1 + c1 + a1)
      . . . . t1 = t1*factor
25    . . . . c1 = c1*factor
      . . . . a1 = a1*factor
      . . . ..end_IF_block
      . . . IF( (g1-G1i)/G1i .gt. Serr)
   Comment  g1 too far above G1i, push it back
30    . . . . g1 = G1i*Fup
      . . . . factor = (1.-g1)/(t1 + c1 + a1)
      . . . . t1 = t1*factor
      . . . . c1 = c1*factor
      . . . . a1 = a1*factor
35    . . . ..end_IF_block
      . . . DO ( a2 = A2i*Fdwn to A2i*Fup in 7 steps)
      . . . . DO ( c2 = C2i*Fdwn to C2i*Fup in 7 steps)
      . . . . . DO (g2=G2i*Fdwn to G2i*Fup in 7 steps)
   Comment     Calc t2 from other concentrations.
40    . . . . . . t2 = 1. - a2 - c2 - g2
      . . . . . . IF( (t2-T2i)/T2i .lt. -Serr)
   Comment  t2 too far below T2i, push it back
      . . . . . . . t2 = T2i*Fdwn
      . . . . . . . factor = (1.-t2)/(a2 + c2 + g2)
45    . . . . . . . a2 = a2*factor
      . . . . . . . c2 = c2*factor
      . . . . . . . g2 = g2*factor
      . . . . . . ..end_IF_block
      . . . . . . IF( (t2-T2i)/T2i .gt. Serr)
50 Comment  t2 too far above T2i, push it back
      . . . . . . . t2 = T2i*Fup
      . . . . . . . factor = (1.-t2)/(a2 + c2 + g2)
```

Table 11, continued.

```
                . . . . . . . a2 = a2*factor
                . . . . . . . c2 = c2*factor
                . . . . . . . g2 = g2*factor
                . . . . . . ..end_IF_block
                . . . . . . IF(g2.gt. 0.0 .and. t2.gt.0.0)
                . . . . . . . t3 = 0.5*(1.-Serr)
                . . . . . . . g3 = 1. - t3
                . . . . . . . CALCULATE-ABUNDANCES
                . . . . . . . COMPARE-ABUNDANCES-TO-PREVIOUS-ONES
                . . . . . . . t3 = 0.5
                . . . . . . . g3 = 1. - t3
                . . . . . . . CALCULATE-ABUNDANCES
                . . . . . . . COMPARE-ABUNDANCES-TO-PREVIOUS-ONES
                . . . . . . . t3 = 0.5*(1.+Serr)
                . . . . . . . g3 = 1. - t3
                . . . . . . . CALCULATE-ABUNDANCES
                . . . . . . . COMPARE-ABUNDANCES-TO-PREVIOUS-ONES
                . . . . . . ..end_IF_block
                . . . . . ..end_DO_loop ! g2
                . . . . ..end_DO_loop ! c2
                . . . ..end_DO_loop ! a2
                . . ..end_DO_loop ! a1
                . ..end_DO_loop ! c1
                ..end_DO_loop ! t1
                WRITE the WORST distribution and the abundances.
```

Table 13: BPTI Homologues

R #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
-3	-	-	-	F	-	-	-	-	-	-	-	-	-	-	-	-	Z	-	-
-2	-	-	-	Q	T	-	-	-	-	-	-	Q	-	-	-	H	G	Z	-
-1	-	-	-	T	E	-	-	-	-	-	-	P	-	-	-	D	D	G	-
1	R	R	R	P	R	R	R	R	R	R	R	L	A	R	R	R	K	R	A
2	P	P	P	P	P	P	P	P	P	P	P	R	K	P	P	P	R	P	A
3	D	D	D	D	D	P	D	D	D	D	D	K	K	D	R	T	D	S	K
4	F	F	F	L	F	D	F	F	F	F	F	L	Y	F	F	F	I	F	Y
5	C	C	C	C	C	F	C	C	C	C	C	I	C	C	E	C	C	N	C
6	L	L	L	Q	L	C	L	L	L	L	L	L	K	E	L	N	R	L	K
7	E	E	E	L	E	L	E	E	E	E	E	H	L	L	P	L	L	P	L
8	P	P	P	P	P	E	P	P	P	P	P	R	P	P	A	P	P	A	P
9	P	P	P	Q	P	P	P	P	Y	Y	P	N	R	A	E	E	P	E	V
10	Y	Y	Y	A	Y	Y	Y	Y	T	T	Y	P	I	E	T	S	E	T	R
11	T	T	T	R	T	T	T	T	G	G	T	G	G	T	G	G	Q	G	Y
12	G	G	G	G	G	G	G	G	P	P	G	R	P	G	L	R	G	P	G
13	P	P	P	P	P	P	P	P	C	C	P	C	C	C	C	C	P	C	C
14	C	C	T	C	K	C	C	C	L	I	C	Y	K	K	K	R	C	K	K
15	K	K	K	K	A	K	G	A	A	A	K	Q	R	A	A	G	K	A	K
16	A	A	A	G	A	A	A	A	A	R	A	K	K	Y	R	H	G	S	K
17	R	R	R	P	A	L	R	R	R	I	R	I	I	I	I	I	R	I	F
18	I	I	I	C	M	L	I	I	I	I	I	P	P	R	R	R	L	R	P
19	I	R	I	K	I	R	R	R	R	R	R	A	S	S	S	I	P	Q	S
20	R	Y	R	A	F	Y	Y	Y	Y	Y	Y	F	F	F	T	Y	R	Y	F
21	Y	F	Y	A	Y	F	F	F	F	F	F	Y	Y	H	H	Y	Y	Y	Y
22	F	Y	F	R	F	Y	Y	Y	Y	Y	Y	Y	T	Y	Y	Y	F	Y	Y
23	Y	N	Y	I	N	N	N	N	N	N	N	N	K	Y	N	N	Y	N	N
24	N	A	N	I	A	K	A	A	A	A	A	Q	W	L	R	L	N	S	W
25	A	K	K	R	K	A	K	K	K	K	K	K	K	A	A	E	P	K	K
26	K	A	A	A	G	G	A	G	A	A	G	K	A	A	A	S	A	S	A
27	G	G	G	G	L	L	G	L	G	G	L	K	K	Q	Q	M	S	G	K
28	L	L	L	L	C	C	L	C	L	L	C	Q	Q	Q	Q	K	R	G	Q
29	C	C	C	C	Q	Q	C	Q	C	C	Q	C	C	C	C	C	M	C	C
30	Q	Q	Q	T	T	T	Q	T	Q	Q	T	E	L	L	L	K	E	Q	Q
31	T	T	T	L	E	T	T	T	T	T	Q	G	P	Q	E	V	S	Q	F
32	F	F	F	P	T	F	F	F	F	F	T	F	F	F	F	F	F	F	I
33	V	V	V	F	F	V	V	V	V	V	V	T	D	I	F	F	I	I	Y
34	Y	Y	Y	T	T	Y	Y	Y	Y	Y	Y	W	Y	Y	I	Y	Y	Y	S
35	G	G	G	G	G	G	G	G	G	G	G	S	S	G	G	G	G	G	G
36	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	C	G
37	C	C	T	A	R	C	C	C	C	C	C	G	G	G	G	G	C	R	G
38	R	R	R	R	A	R	R	R	R	R	R	G	G	G	G	G	K	G	G
39	A	A	A	A	K	A	A	A	A	A	A	N	N	N	N	N	G	N	N
40	K	K	K	K	S	K	K	K	K	K	K	S	A	A	A	A	N	Q	A
41	R	R	R	N	N	R	R	R	R	R	R	N	N	N	N	N	K	N	N
42	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
43																			

Table 13, continued.

R #	20	21	22	23	24	25	26	27	28	29	30	31	32	33
-5	-	-	-	-	-	-	-	-	-	-	-	-	-	D
-4	-	-	-	-	-	-	-	-	-	-	-	-	-	E
-3	-	-	-	-	-	-	-	-	-	-	-	-	T	P
-2	Z	-	L	Z	R	K	-	-	-	R	R	-	E	T
-1	P	-	Q	D	D	N	-	-	-	Q	K	-	R	T
1	R	R	H	H	R	R	I	K	T	R	R	R	G	D
2	R	P	R	P	P	P	N	E	V	H	H	P	F	L
3	K	Y	T	K	K	T	G	D	A	R	P	D	L	P
4	L	A	F	F	F	F	D	S	A	D	D	F	D	I
5	C	C	C	C	C	C	C	C	C	C	C	C	C	C
6	I	E	K	Y	Y	N	E	Q	N	D	D	L	T	E
7	L	L	L	L	L	L	L	L	L	K	K	E	S	Q
8	H	I	P	P	P	L	P	G	P	P	P	P	P	A
9	R	V	A	A	A	P	K	Y	V	P	P	P	P	FG
10	N	A	E	D	D	E	V	S	I	D	D	Y	V	D
11	P	A	P	P	P	T	V	A	R	K	T	T	T	A
12	G	G	G	G	G	G	G	G	G	G	K	G	G	G
13	R	P	P	R	R	R	P	P	P	N	I	P	P	L
14	C	C	C	C	C	C	C	C	C	C	C	C	C	C
15	Y	M	K	K	L	N	R	M	R	-	-	K	R	F
16	D	F	A	A	A	A	A	G	A	G	Q	A	A	G
17	K	F	S	H	Y	L	R	M	F	P	T	K	G	Y
18	I	I	I	I	M	I	F	T	I	V	V	M	F	M
19	P	S	P	P	P	P	P	S	Q	R	R	I	K	K
20	A	A	A	R	R	A	R	R	L	A	A	R	R	L
21	F	F	F	F	F	F	Y	Y	W	F	F	Y	Y	Y
22	Y	Y	Y	Y	Y	Y	Y	F	A	Y	Y	F	N	S
23	Y	Y	Y	Y	Y	Y	Y	Y	F	Y	Y	Y	Y	Y
24	N	S	N	D	N	N	N	N	D	D	K	N	N	N
25	Q	K	W	S	P	S	S	G	A	T	P	A	T	Q
26	K	G	A	A	A	H	S	T	V	R	S	K	R	E
27	K	A	A	S	S	L	S	S	K	L	A	A	T	T
28	K	N	K	N	N	H	K	M	G	K	K	G	K	K
29	Q	K	K	K	K	K	R	A	K	T	R	F	Q	N
30	C	C	C	C	C	C	C	C	C	C	C	C	C	C
31	E	Y	Q	N	E	Q	E	E	V	K	V	E	E	E
32	R	P	L	K	K	K	K	T	L	A	Q	T	P	E
33	F	F	F	F	F	F	F	F	F	F	F	F	F	F
34	D	T	H	I	I	N	I	Q	P	Q	R	V	K	I
35	W	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
36	S	S	G	G	G	G	G	G	G	R	G	G	G	G
37	G	G	G	G	G	G	G	G	G	G	G	G	G	G
38	C	C	C	C	C	C	C	C	C	C	C	C	C	C
39	G	R	K	P	R	G	G	M	Q	D	D	K	K	Q
40	G	G	G	G	G	G	G	G	G	G	G	A	G	G
41	N	N	N	N	N	N	N	N	N	D	D	K	N	N
42	S	A	A	A	A	A	A	G	G	H	H	S	G	D
43	N	N	N	N	N	N	N	N	N	G	G	N	N	N

Table 13, continued.

R #	20	21	22	23	24	25	26	27	28	29	30	31	32	33
44	R	R	R	N	N	N	N	N	K	N	N	N	R	R
45	F	F	F	F	F	F	F	F	F	F	F	F	Y	F
46	K	K	S	K	K	K	H	V	Y	K	K	R	K	S
47	T	T	T	T	T	T	T	T	S	T	S	S	S	T
48	I	I	I	W	W	I	L	E	E	E	D	A	E	L
49	E	E	E	D	D	D	E	K	K	T	H	E	Q	A
50	E	E	K	E	E	E	E	E	E	L	L	D	D	E
51	C	C	C	C	C	C	C	C	C	C	C	C	C	C
52	R	R	R	R	R	Q	E	L	R	R	R	M	L	E
53	R	R	H	Q	H	R	K	Q	E	C	C	R	D	Q
54	T	T	A	T	T	T	V	T	Y	E	E	T	A	K
55	C	C	C	C	C	C	C	C	C	C	C	C	C	C
56	I	V	V	G	V	A	G	R	G	L	E	G	S	I
57	G	V	G	A	A	A	V	-	V	V	L	G	G	N
58	-	-	-	S	S	K	R	-	P	Y	Y	A	F	-
59	-	-	-	A	G	Y	S	-	G	P	R	-	-	-
60	-	-	-	-	I	G	-	-	D	-	-	-	-	-

20 Dendroaspis angusticeps (Eastern Green Mamba) C13 S2 C3 toxin (DUFT85)
21 Dendroaspis polylepis polylepes (Black mamba) B toxin (DUFT85)
22 Dendroaspis polylepis polylepes (Black Mamba) E toxin (DUFT85)
23 Vipera ammodytes TI toxin (DUFT85)
24 Vipera ammodytes CTI toxin (DUFT85)
25 Bungarus fasciatus VIII B toxin (DUFT85)
26 Anemonia sulcata (sea anemone) 5 II (DUFT85)
27 Homo sapiens HI-14 "inactive" domain (DUFT85)
28 Homo sapiens HI-14 "active" domain (DUFT85)
29 beta bungarotoxin B1 (DUFT85)
30 beta bungarotoxin B2 (DUFT85)
31 Bovine spleen TI II (FIORS5)
32 Tachypleus tridentatus (Horseshoe crab) hemocyte inhibitor (NAKA87)
33 Bombyx mori (silkworm) SCI-III (SASA84)

Notes :
a) both beta bungarotoxins have residue 15 deleted.
b) B. mori has an extra residue between C5 and C14; we have assigned F and G to residue 9.
c) all natural proteins have C at 5, 14, 30, 38, 50, & 55.
d) all homologues have F33 and G37.
e) extra C's in bungarotoxins form interchain cystine bridges

Table 14: Tally of Ionizable Groups.
BPTI homologues.

Sequence Identifier	D	E	K	R	Y	H	NH	CO2	+	#
1	2	2	4	6	4	0	1	1	6	16
2	2	2	4	6	4	0	1	1	6	16
3	2	2	4	6	4	0	1	1	6	16
4	2	4	2	3	3	0	1	1	-1	13
5	2	4	4	4	4	0	1	1	2	16
6	2	2	3	6	4	0	1	1	5	15
7	2	2	3	6	4	0	1	1	5	15
8	2	2	3	6	4	0	1	1	5	15
9	2	2	3	6	4	0	1	1	5	15
10	2	2	3	6	4	0	1	1	5	15
11	2	3	4	6	4	0	1	1	5	19
12	0	3	7	7	3	1	1	1	11	19
13	1	2	8	5	4	0	1	1	10	18
14	2	3	2	5	3	1	1	1	2	14
15	1	4	2	7	2	2	1	1	4	16
16	2	5	3	7	3	2	1	1	3	19
17	2	4	6	7	3	0	1	1	7	21
18	1	1	2	4	4	0	1	1	4	8
19	0	2	9	4	4	0	1	1	11	17
20	2	3	6	7	3	1	1	1	8	20
21	0	3	3	5	5	0	1	1	5	13
22	0	2	6	3	3	2	1	1	7	13
23	4	1	5	3	4	2	1	1	3	15
24	3	2	4	6	5	1	1	1	5	17
25	1	2	5	3	3	1	1	1	5	13
26	1	5	4	4	4	1	1	1	2	16
27	1	4	2	2	4	0	1	1	-1	11
28	2	3	4	3	3	0	1	1	2	14
29	6	2	5	7	4	2	1	1	4	22
30	6	2	6	7	4	2	1	1	5	23
31	2	3	5	4	4	0	1	1	4	16
32	3	3	5	5	4	0	1	1	4	18
33	4	7	3	1	4	0	1	1	-7	17

Sequences given in Table 10.

+ is sum of K + R + NH - D - E - CO2, approximate charge on molecule at pH 7.0

is sum of K + R + NH + D + E + CO2, i.e. number of ionized groups at pH 7.0.

Table 15: Amino acids observed at each Residue BPTI homologues

Res. #	Number Differnet AAs	Contents	BPTI
-5	2	D -32	-
-4	2	E -32	-
-3	5	T P F Z -29	-
-2	10	Z3 R3 Q2 T2 H G L K E -18	-
-1	10	D4 T2 P2 Q2 E G N K R -18	-
1	10	R21 A2 K2 H2 P L I T G D	R
2	9	P20 R4 A2 H2 N E V F L	P
3	10	D15 K6 T3 R2 P2 S Y G A L	D
4	7	F19 D4 L3 Y2 I2 A2 S	F
5	1	C33	C
6	10	L11 E5 N4 K3 Q2 I2 Y2 D2 T R	L
7	5	L18 E11 K2 S Q	E
8	7	P26 H2 A2 I L G F	P
9	9	P17 A6 V3 R2 Q L K Y F	P
10	10	Y11 E7 D4 A2 N2 R2 V2 S I D	Y
11	10	T17 P5 A3 R2 I S Q Y V K	T
12	2	G32 K	G
13	5	P22 R6 L3 N I	P
14	3	C31 T A	C
15	12	K15 R4 Y2 M2 L2 -2 V G A I N F	K
16	7	A22 G5 Q2 R K D F	A
17	12	R12 K5 A2 Y3 H2 S2 F2 L M T G P	R
18	6	I21 M4 F3 L2 V2 T	I
19	7	I11 P10 R6 S2 K2 L Q	I
20	5	R19 A7 S4 L2 Q	R
21	4	Y18 F13 W I	Y
22	6	F14 Y14 H2 A N S	F
23	2	Y32 F	Y
24	4	N26 K3 D3 S	N
25	10	A12 S5 Q3 P3 W3 L2 T2 K G R	A
26	9	K16 A6 T2 E2 S2 R2 G H V	K
27	5	A18 S8 K3 L2 T2	A
28	7	G13 K10 N5 Q2 R H M	G
29	10	L9 Q7 K7 A2 F2 R2 M G T N	L
30	1	C33	C
31	7	Q12 E11 L4 K2 V2 Y N	Q
32	11	T12 P5 K4 Q3 E2 L2 G V S R A	T
33	1	F33	F
34	11	V11 I8 T3 D2 N2 Q2 F H P R K	V
35	2	Y31 W2	Y
36	3	G27 S5 R	G
37	1	G33	G
38	3	C31 T A	C
39	7	R13 G9 K4 Q3 D2 P M	R

Table 15: continued.

Res. #	Number Different AAs	Contents	
40	2	G22 A11	A
41	3	N20 K11 D2	K
42	9	A11 R9 S4 G3 H2 D Q K N	R
43	2	N31 G2	N
44	3	N21 R11 K	N
45	2	F32 Y	F
46	8	K24 E2 S2 D H V Y R	K
47	2	T19 S14	S
48	9	A11 I9 E4 T2 W2 L2 R K D	A
49	7	E19 D6 A2 Q2 K2 T H	E
50	6	E16 D12 L2 M Q K	D
51	1	C33	C
52	7	R13 M10 L3 E3 Q2 H V	M
53	8	R21 Q3 E2 H2 C2 G K D	R
54	7	T23 A3 V2 E2 I Y K	T
55	1	C33	C
56	8	G15 V8 I3 E2 R2 A L S	G
57	8	G19 V4 A3 P2 -2 R L N	G
58	8	A11 -10 P3 K3 S2 Y2 R F	A
59	9	-24 G2 Q E A Y S P R	-
60	6	-28 Q R I G D	-
61	3	-31 T P	-
62	2	-32 D	-
63	2	-32 K	-
64	2	-32 S	-

Table 16: Exposure in BPTI

Coordinates taken from
Brookhaven Protein Data Bank entry 6PTI.

```
HEADER    PROTEINASE INHIBITOR (TRYPSIN) 13-MAY-87   6PTI
COMPND    BOVINE PANCREATIC TRYPSIN INHIBITOR
COMPND   2(/BPTIS,CRYSTAL FORM /IIIS)
AUTHOR    A.WLODAWER
```

Solvent radius = 1.40
Atomic radii given in Table 7

Areas in Angstroms-squared.

Residue	Total area	Not Covered by M/C	fraction	Not covered at all	fraction
ARG 1	342.45	205.09	0.5989	152.49	0.4453
PRO 2	239.12	92.65	0.3875	47.56	0.1989
ASP 3	272.39	158.77	0.5829	143.23	0.5258
PHE 4	311.33	137.82	0.4427	43.21	0.1388
CYS 5	241.06	48.36	0.2006	0.23	0.0010
LEU 6	280.98	151.45	0.5390	115.87	0.4124
GLU 7	291.39	128.91	0.4424	90.39	0.3102
PRO 8	236.12	128.71	0.5451	99.98	0.4234
PRO 9	236.09	109.82	0.4652	45.80	0.1940
TYR 10	330.97	153.63	0.4642	79.49	0.2402
THR 11	249.20	80.10	0.3214	64.99	0.2608
GLY 12	184.21	56.75	0.3081	23.05	0.1252
PRO 13	240.07	130.25	0.5426	75.27	0.3136
CYS 14	237.10	75.55	0.3186	53.52	0.2257
LYS 15	310.77	200.25	0.6444	192.00	0.6178
ALA 16	209.41	66.63	0.3182	45.59	0.2177
ARG 17	351.09	243.67	0.6940	201.48	0.5739
ILE 18	277.10	100.51	0.3627	58.95	0.2127
ILE 19	278.03	146.06	0.5254	96.05	0.3455
ARG 20	339.11	144.65	0.4266	43.81	0.1292
TYR 21	333.60	102.24	0.3065	69.67	0.2089
PHE 22	306.08	70.64	0.2308	23.01	0.0752
TYR 23	338.66	77.05	0.2275	17.34	0.0512
ASN 24	264.88	99.03	0.3739	38.69	0.1461
ALA 25	211.15	85.13	0.4032	48.20	0.2283
LYS 26	313.29	216.14	0.6899	202.84	0.6474
ALA 27	210.66	96.05	0.4560	54.78	0.2601
GLY 28	186.83	71.52	0.3828	32.09	0.1718
LEU 29	280.70	132.42	0.4718	93.61	0.3335
CYS 30	238.15	57.27	0.2405	19.33	0.0812
GLN 31	301.15	141.80	0.4709	82.64	0.2744
THR 32	251.26	138.17	0.5499	76.47	0.3043

Table 16, continued.

PHE 33	304.27	59.79	0.1965	18.91	0.0622
VAL 34	251.56	109.78	0.4364	42.36	0.1684
TYR 35	332.64	80.52	0.2421	15.05	0.0452
GLY 36	187.06	11.90	0.0636	1.97	0.0105
GLY 37	185.28	84.26	0.4548	39.17	0.2114
CYS 38	234.56	73.64	0.3139	26.40	0.1125
ARG 39	417.13	304.62	0.7303	250.73	0.6011
ALA 40	209.53	94.01	0.4487	52.95	0.2527
LYS 41	314.60	166.23	0.5284	108.77	0.3457
ARG 42	349.06	232.83	0.6670	179.59	0.5145
ASN 43	266.47	38.53	0.1446	5.32	0.0200
ASN 44	269.65	91.08	0.3378	23.39	0.0867
PHE 45	313.22	69.73	0.2226	14.79	0.0472
LYS 46	309.83	217.18	0.7010	155.73	0.5026
SER 47	224.78	69.11	0.3075	24.80	0.1103
ALA 48	211.01	82.06	0.3889	31.07	0.1473
GLU 49	286.62	161.00	0.5617	100.01	0.3489
ASP 50	299.53	156.42	0.5222	95.96	0.3204
CYS 51	238.68	24.51	0.1027	0.00	0.0000
MET 52	293.05	89.48	0.3054	66.70	0.2276
ARG 53	356.20	224.61	0.6306	189.75	0.5327
THR 54	251.53	116.43	0.4629	51.64	0.2053
CYS 55	240.40	69.95	0.2910	0.00	0.0000
GLY 56	184.66	60.79	0.3292	32.78	0.1775
GLY 57	106.58	49.71	0.4664	38.26	0.3592
ALA 58	no position given in Protein Data Bank				

"Total area" is the area measured by a rolling sphere of radius 1.4 A, where only the atoms within the residue are considered. This takes account of conformation.

"Not covered by M/C" is the area measured by a rolling sphere of radius 1.4 A where all main-chain atoms are considered, fraction is the exposed area divided by the total area. Surface buried by main-chain atoms is more definitely covered than is surface covered by side group atoms.

"Not covered at all" is the area measured by a rolling sphere of radius 1.4 A where all atoms of the protein are considered.

Table 17: Plasmids used in Detailed Example

Phage	Contents
LG1	M13mp18 with Ava II/Aat II/Acc I/Rsr II/Sau I adaptor
pLG2	LG1 with amp^R and ColE1 of pBR322 cloned into Aat II/Acc I sites
pLG3	pLG2 with Acc I site removed
pLG4	pLG3 with first part of osp-pbd gene cloned into Rsr II/Sau I sites, Avr II/Asu II sites created
pLG5	pLG4 with second part of osp-pbd gene cloned into Avr II/Asu II sites, HasH I site created
pLG6	pLG5 with third part of osp-pbd gene cloned into Asu II/BasH I sites, Bbe I site created
pLG7	pLG6 with last part of osp-pbd gene cloned into Bbe I/Asu II sites
pLG8	pLG7 with disabled osp-pbd gene, same length DNA.
pLG9	pLG7 mutated to display BPTI($V15_{BPTI}$)
pLG10	pLG8 + tet^R gene - amp^R gene
pLG11	pLG9 + tet^R gene - amp^R gene

Table 18: Enzyme sites eliminated when M13mp18 is cut by Ava II and BsuJ6 I

Aha II	Nar I	Gdi II	Pvu I
Fsp I	Bgl I	HgiE II	BsuJ6 I
EcoR I	Sac I	Kpn I	Xma I
Sma I	BamH I	Xba I	Sal I
Hind III	Acc I	Pst I	Sph I
Hind II			

Table 19: Enzymes not cutting M13mp18

Aat II	Afl I	Apa I	Avr II
Bbv II	Bcl I	BspM I	BssH I
BstB I	BstE II	BstX I	Eag I
Eco57 I	EcoN I	Eco0109 I	EcoR V
Esp I	Hpa I	Mlu I	Nco I
Nhe I	Not I	Nru I	Nsi I
PflM I	PmaC I	Ppa I	PpuM I
Rsr I	Sac I	Sca I	Sfi I
Spe I	Stu I	Sty I	Tth111 I
Xca I	Xho I		

Table 20: Enzymes cutting AmpR gene and ori

	Aat II	Bbv II	Eco57 I	Ppa I
	Sca I	Tth111 I	Aha II	Gdi II
	Pvu I	Fsp I	Bql I	HgiE II
	Hind II	Pst I	Xba I	Afl III
	Nde I			

Table 21: Enzymes tested on Aeblq DNA

Enzyme	Recognition	Symm	cuts		Supply
%Acc I	GTMKAC	P	2	4	<B,M,I,N,P,T
Afl II	CTTAAG	P	1	5	<N
Apa I	GGGCCC	P	5	1	<M,I,N,P,T
Asu II	TTCGAA	P	2	4	<P,N(BstB I)
Ava III	ATGCAT	P	5	1	<T; Nsi I:M,N,P,T; EcoT22 I:T
Avr II	CCTAGG	P	1	5	<N
BamH I	GGATCC	P	1	5	<S,B,M,I,N,P,T
Bcl I	TGATCA	P	1	5	<S,B,M,I,N,T
BspM II	TCCGGA	P	1	5	<N
BssH II	GCGCGC	P	1	5	<N,T
+BstE II	GGTNACC	P	1	6	<S,B,M,N,T
%BstX I	CCANNNNNN	P	8	4	<N,P,T
+Dra II	RGGNCCY	P	2	5	<M,T ; Eco0109 I:N
+EcoN I	CCTNNNNNN	P	5	6	<N(soon)
EcoR I	GAATTC	P	1	5	<S,B,M,I,N,P,T
EcoR V	GATATC	P	3	3	<S,B,M,I,N,P,T
+Esp I	GCTNAGC	P	2	5	<T
Hind III	AAGCTT	P	1	5	<S,B,M,I,N,P,T
Hpa I	GTTAAC	P	3	3	<S,B,M,I,N,P ,T
Kpn I	GGTACC	P	5	1	<S,B,M,I,N,P,T ; Asp718:M
Mlu I	ACGCGT	P	1	5	<M,N,P,T
Nar I	GGCGCC	P	2	4	<B,N,T
Nco I	CCATGG	P	1	5	<B,M,N,P,T
Nhe I	GCTAGC	P	1	5	<M,N,P,T
Not I	GCGGCCGC	P	2	6	<M,N,P,T
Nru I	TCGCGA	P	3	3	<B,M,N,T
+PflM I	CCANNNNTN	P	7	4	<N
PmaC I	CACGTG	P	3	3	<none
+PpuM I	RGGWCCY	P	2	5	<N
+Rsr II	CGGWCCG	P	2	5	<N,T
Sac I	GAGCTC	P	5	1	<B(Sst I),M,I,N,P,T
Sal I	GTCGAC	P	1	5	<B,M,I,N,P,T
+Sau I	CCTNAGG	P	2	5	<M; Cvn I:B; Mse I:T; Bsu15 I:N; Aoc I:T
+Sfi I	GGCCNNNNNGGCC	P	8	5	<N,P,T
Sma I	CCCGGG	P	3	3	<B,M,I,N,P,T
Spe I	ACTAGT	P	1	5	<M,N,T
Sph I	GCATGC	P	5	1	<B,M,I,N,P,T
Stu I	AGGCCT	P	3	3	<M,N,I(Aat I),P,T
%Stv I	CCWWGG	P	1	5	<N,P,T
Vca I	GTATAC	P	3	3	<N(soon)

Table 21, continued.

5	Xho I	CTCGAG	P	1	& 5	<B,M,I,P,T: Ccr I: T ; PaeR7 I:N
	Xma I	CCCGGG	P	1	& 5	<I,N,P,T
	Xma III	CGGCCG	P	1	& 5	<B; Eag I:N; Eco52 I:T

10

N_restrct = 43

15

Table 22: ipbd gene

```
pbd mod10 29III88 :
   lacUV5 Rsr II/Avr II/gene/TrpA attenuator/Mst II; !
5'-    CGGaCCG TaT                              ! Rsr II site
CCAGGC tttaca CTTTATGCTTCCGGCTCG tataat GTG ! lacUV5
TGG aATTGTGAGCGGATAACAATT                       ! lacO opersator
CCT AGGAgg CtcaCT                               ! Shine-Dalgarno seq.
atg aag aaa tct ctg gtt ctt aag gct agc ! 10, Mi3 leader
gtt gct gtc gcg acc ctg gta ccg atg ctg ! 20
tct ttt gct cgt ccg gat ttc tgt ctc gag ! 30
ccg cca tat act ggg ccc tgc aaa gcg cgc ! 40
atc atc cgt tat ttc tac aac gct aaa gca ! 50
ggc ctg tgc cag acc ttt gta tac ggt ggt ! 60
tgc cgt gct aag cgt aac aac ttt aaa tcg ! 70
gcc gaa gat tgc atg cgt acc tgc ggt ggc ! 80
gcc gct gaa ggt gat gat ccg gcc aaa gcg ! 90
gcc ttt aac tct ctg caa gct tct gct acc ! 100
gaa tat atc ggt tac gcg tgg gcc atg gtg ! 110
gtg gtt atc gtt ggt gct acc atc ggt atc ! 120
aaa ctg ttt aag aaa ttt act tcg aaa gcg ! 130
tct taa tag tga ggttacc !   BstE II
agtcta agcccgc ctaatga gcgggct tttttttt ! terminator
CCTgAGG            -3'  ! Mst II
```

Table 23: ipbd DNA sequence

DNA Sequence file = UV5_M13PTIM13.DNA;17

DNA Sequence title =

pbd mod10 29III88 : lac-UV5 RsrII/AvrII/gene/TrpA attenuator/MstII; !

```
  1   C|GGA|CCG|TAT|CCA|GGC|TTT|ACA|CTT|TAT|GCT|TCC|GGC|TCG|
 41 TAT|AAT|GTG|TGG|AAT|TGT|GAG|CGG|ATA|ACA|ATT|CCT|AGG|AGG|
 83 CTC|ACT|ATG|AAG|AAA|TCT|CTG|GTT|CTT|AAG|GCT|AGC|GTT|GCT|
125 GTC|GCG|ACC|CTG|GTA|CCG|ATG|CTG|TCT|TTT|GCT|CGT|CCG|GAT|
167 TTC|TGT|CTC|GAG|CCG|CCA|TAT|ACT|GGG|CCC|TGC|AAA|GCG|CGC|
209 ATC|ATC|CGT|TAT|TTC|TAC|AAC|GCT|AAA|GCA|GGC|CTG|TGC|CAG|
251 ACC|TTT|GTA|TAC|GGT|GGT|TGC|CGT|GCT|AAG|CGT|AAC|AAC|TTT|
293 AAA|TCG|GCC|GAA|GAT|TGC|ATG|CGT|ACC|TGC|GGT|GGC|GCC|GCT|
335 GAA|GGT|GAT|GAT|CCG|GCC|AAA|GCG|GCC|TTT|AAC|TCT|CTG|CAA|
377 GCT|TCT|GCT|ACC|GAA|TAT|ATC|GGT|TAC|GCG|TGG|GCC|ATG|GTG|
419 GTG|GTT|ATC|GTT|GGT|GCT|ACC|ATC|GGT|ATC|AAA|CTG|TTT|AAG|
461 AAA|TTT|ACT|TCG|AAA|GCG|TCT|TAA|TAG|TGA|GGT|TAC|CAG|TCT|
503 AAG|CCC|GCC|TAA|TGA|GCG|GGC|TTT|TTT|TTT|CCT|GAG|G
```

Total = 539 bases

Table 24: Summary of Restriction Cuts

```
Enz = %Acc I  has   1 observed sites : 259
Enz = Acc III  has   1 observed sites : 162
Enz = Acy I  has   1 observed sites : 328
Enz = Afl II  has   1 observed sites : 109
Enz = %Afl III  has   1 observed sites : 404
Enz = Aha III  has   1 observed sites : 292
Enz = Apa I  has   1 observed sites : 193
Enz = Aso718  has   1 observed sites : 138
Enz = Asu II  has   1 observed sites : 471
Enz = %Ava I  has   1 observed sites : 175
Enz = Avr II  has   1 observed sites :  76
Enz = %Ban I  has   3 observed sites : 138 328 540
Enz = Bbe I  has   1 observed sites : 328
Enz = +Bgl I  has   1 observed sites : 352
Enz = +Bin I  has   1 observed sites : 346
Enz = %BspM I  has   1 observed sites : 319
Enz = BssH II  has   1 observed sites : 205
Enz = +BstE II  has   1 observed sites : 493
Enz = %BstX I  has   1 observed sites : 413
Enz = Cfr I  has   2 observed sites : 299 350
Enz = +Dra II  has   1 observed sites : 193
Enz = +Esp I  has   1 observed sites : 277
Enz = %Fok I  has   1 observed sites : 213
Enz = Gdi II  has   2 observed sites : 299 350
Enz = Hae I  has   1 observed sites : 240
Enz = Hae II  has   1 observed sites : 328
Enz = +Hga I  has   1 observed sites : 478
Enz = %HgiC I  has   3 observed sites : 138 328 540
Enz = %HgiJ II  has   1 observed sites : 193
Enz = Hind III  has   1 observed sites : 377
Enz = +Hph I  has   1 observed sites : 340
Enz = Kpn I  has   1 observed sites : 138
Enz = +Mbo II  has   2 observed sites :  93 304
Enz = Mlu I  has   1 observed sites : 404
Enz = Nar I  has   1 observed sites : 328
Enz = Nco I  has   1 observed sites : 413
Enz = Nhe I  has   1 observed sites : 115
Enz = Nru I  has   1 observed sites : 128
Enz = Nsp(7524)  has   1 observed sites : 311
Enz = NspB II  has   1 observed sites : 332
Enz = +PflM I  has   1 observed sites : 184
Enz = +Pss I  has   1 observed sites : 193
Enz = +Rsr II  has   1 observed sites :   3
Enz = +Sau I  has   1 observed sites : 535
Enz = %SfaN I  has   2 observed sites : 144 209
Enz = +Sfi I  has   1 observed sites : 351
Enz = Sph I  has   1 observed sites : 311
Enz = Stu I  has   1 observed sites : 240
Enz = %Sty I  has   2 observed sites :  76 413
```

Table 24, continued.

Enz = Xca I has 1 observed sites : 259
Enz = Xho I has 1 observed sites : 175
Enz = Xma III has 1 observed sites : 299

Enzymes that do not cut

Aat II	AlwN I	ApaL I	Ase I	Ava III
Bal I	BamH I	Bbv I	Bbv II	Bcl I
Bgl II	Bsm I	BspH I	Cla I	Dra III
Eco47 III	EcoN I	EcoR I	EcoR V	HgiA I
Hinc II	Hpa I	Mst I	Nae I	Nde I
Not I	Ple I	PnaC I	PpuM I	Pst I
Pvu I	Pvu II	Sac I	Sac II	Sal I
Sca I	Sma I	SnaB I	Spe I	Ssp I
Tag II	Tth111 I	Tth111 II	Xho II	Xma I
Xmn I				

Table 25: Annotated Sequence of ipbd gene

```
5'- C|GGA|CCG|TAT|CCA|GGC|TTT|ACA|CTT|TAT|                          28
   | Rsr II |          |  -35  |

|GCT|TCC|GGC|TCG|TAT|AAT|GTG|TGG|                                  52
              | -10 |

|AAT|TGT|GAG|CGG|ATA|ACA|ATT|                                      73
   lac operator

|CCT|AGG|AGG|CTC|ACT|                                              88
 Avr II
   | S. D. |

| m | k | k | s | l | v | l | k | a | s |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10|
|ATG|AAG|AAA|TCT|CTG|GTT|CTT|AAG|GCT|AGC|                          118
                        | Af| II| Nhe I |

| v | a | v | a | t | l | v | p | n | l |
| 11| 12| 13| 14| 15| 16| 17| 18| 19| 20|
|GTT|GCT|GTC|GCG|ACC|CTG|GTA|CCG|ATG|CTG|                          148
        | Nru I |       | Xon I |

| s | f | a | r | p | d | f | c | l | e |
| 21| 22| 23| 24| 25| 26| 27| 28| 29| 30|
|TCT|TTT|GCT|CGT|CCG|GAT|TTC|TGT|CTC|GAG|                          178
            |AccIII|          Ava I |
                                | Xho I |

| p | p | y | t | q | p | c | k | a | r |
| 31| 32| 33| 34| 35| 36| 37| 38| 39| 40|
|CCG|CCA|TAT|ACT|GGG|CCC|TGC|AAA|GCG|CGC|                          208
    PflM I |          |          | BssH II |
           | Apa I |
           | Dra II |
           | Pss I |

| i | i | r | y | f | y | n | a | k |
| 41| 42| 43| 44| 45| 46| 47| 48| 49|
|ATC|ATC|CGT|TAT|TTC|TAC|AAC|GCT|AAA|                              235
```

Table 25, continued.

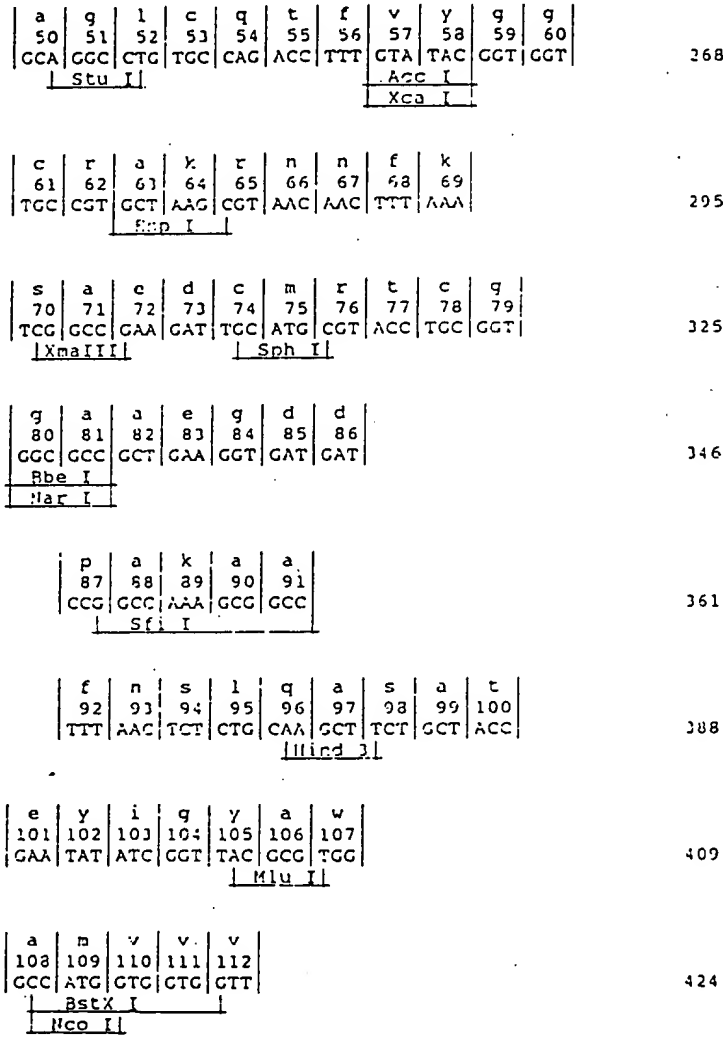

Table 25, continued.

i	v	g	a	t	i	g	i	
113	114	115	116	117	118	119	120	
ATC	GTT	GGT	GCT	ACC	ATC	GGT	ATC	448

k	l	f	k	k	f	t	s	k	a	
121	122	123	124	125	126	127	128	129	130	
AAA	CTG	TTT	AAG	AAA	TTT	ACT	TCG	AAA	GCG	478

Asu III

s	.	.	.					
131	132	133	134					
TCT	TAA	TAG	TGA	GGT	TAC	CAG	TCT	502

BstE II

AAG	CCC	GCC	TAA	TGA	GCG	GGC	TTT	TTT	TTT	532

Trp terminator

CCT	GAG	G -3'	539

Sau I

Note the following enzyme equivalences,

Xma III = Eag I
Acc III = BspM II
Pra II = Eco0109 I
Asu II = BstB I
Sau I = Bsu36 I

Table 26: DNA_seq1

```
5' |ccg|tcc|gtC|GGA|CCG|TAT|CCA|GGC|TTT|ACA|CiT|TAT|
    spacer  | Rsr II |          |  -35  |

|GCT|TCC|GGC|TCG|TAT|AAT|GTG|TGG|
              | -10 |

    |AAT|TGT|GAG|CGG|ATA|ACA|ATT|
      lac operator

    |CCT|AGG|
     Avr II

                                  s   k   a
                                 128 129 130
                |gcc|gct|ccT|TCG|AAA|GCG|
                  spacer   |Asu II|

 s
131 .   .   .
TCT TAA TAG TGA |GGT|TAC|CAG|TCT|
                 | BstE II|

|AAG|CCC|GCC|TAA|TGA|GCG|GGC|TTT|TTT|TTT|
   | Trp terminator                      |

|CCT|GAG|Gca|ggt|gag|cg - 3'
 Sau I  | spacer     |
```

Table 27: DNA_synth1

```
5' |CCG|TCC|GTC|GGA|CCG|TAT|CCA|GGC|TTT|ACA|CTT|TAT|

|GCT|TCC|GGC|TCG|TAT|AAT|GTG|TGG|

    |AAT|TGT|GAG|CGG|ATA|ACA|ATT|
          olig#4 = 3'- gt taa

    |CCT|AGG|
     gga tcc

                                  / 3' = olig#3
              |GCC|GCT|CCT|TCG|AAA|GCG|
               cgg cga gga agc ttt cgc

|TCT|TAA|TAG|TGA|GGT|TAC|CAG|TCT|
 aga att atc act cca atg gtc aga

|AAG|CCC|GCC|TAA|TGA|GCG|GGC|TTT|TTT|TTT|
 ttc ggg cgg att act cgc ccg aaa aaa aaa

|CCT|GAG|GCA|GGT|GAG|CG
 gga ctc cgt cca ctc gc - 5'
```

"Top" strand	99	
"Bottom" strand	100	
Overlap	23	(14 c/g and 9 a/t)
Net length	158	

Table 28: DNA_seq2

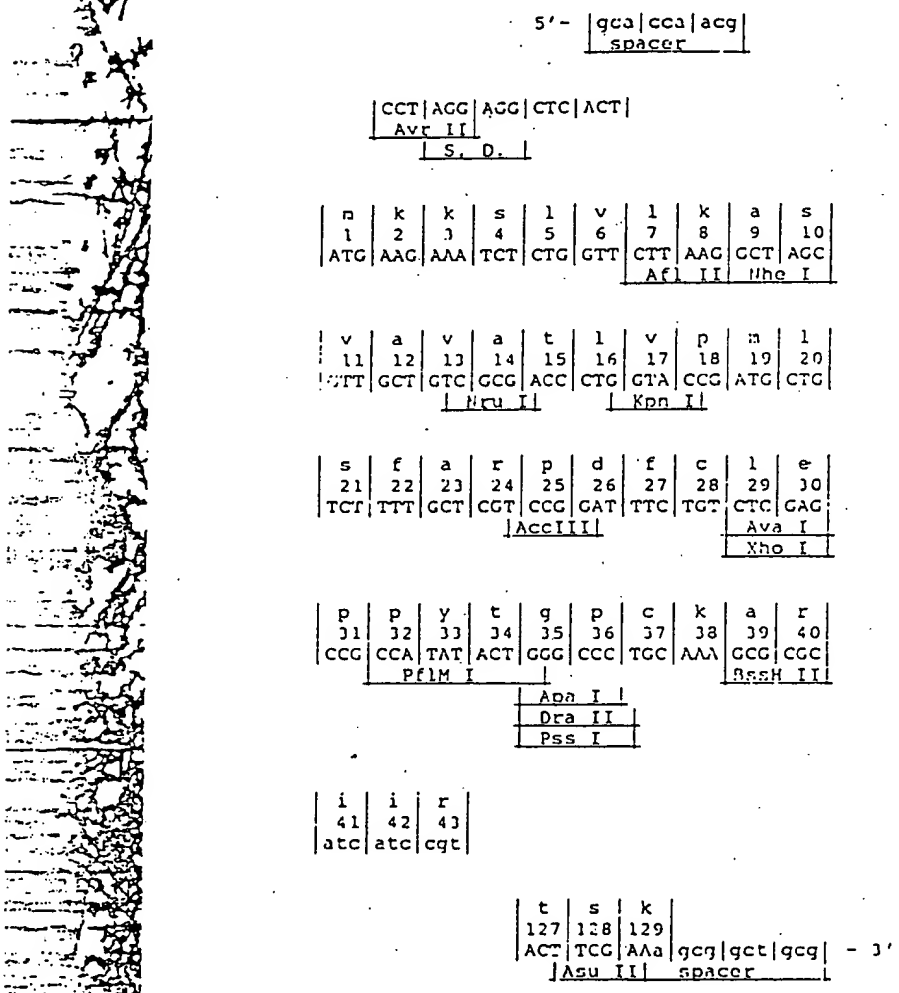

Table 29: DNA_synth2

5'- |GCA|CCA|ACG|

|CCT|AGG|AGG|CTC|ACT|

|ATG|AAG|AAA|TCT|CTG|GTT|CTT|AAG|GCT|AGC|

|GTT|GCT|GTC|GCG|ACC|CTG|GTA|CCG|ATG|CTG|
olig#6 = 3'- ggc tac gac

/ 3' = olig#5
|TCT|TTT|GCT|CGT|CCG|GAT|TTC|TGT|CTC|GAG|
aga aaa cga gca ggc cta aag aca gag ctc

|CCG|CCA|TAT|ACT|GGG|CCC|TGC|AAA|GCG|CGC|
ggc ggt ata tga ccc ggg acg ttt cgc gcg

|ATC|ATC|CGT|
tag tag gca

|ACT|TCG|AAA|GCG|GCT|GCG|
tga agc ttt cgc cga cgc - 5'

"Top" strand	99	
"Bottom" strand	99	
Overlap	24	(14 c/g and 10 a/t)
Net-length	155	

Table 30: DNA_seq3

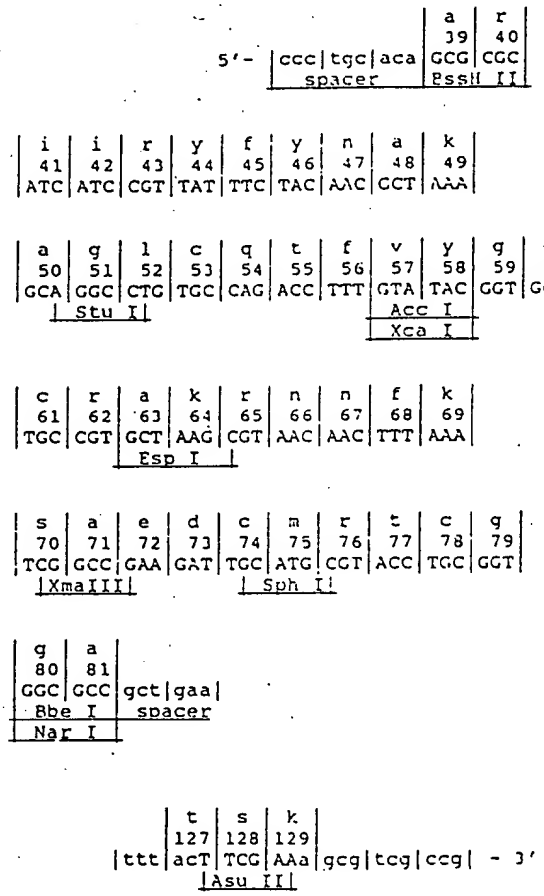

Table 31: DNA_synth3

```
5'-|CCC|TGC|ACA|GCG|CGC|

|ATC|ATC|CGT|TAT|TTC|TAC|AAC|GCT|AAA|

|GCA|GGC|CTG|TGC|CAG|ACC|TTT|GTA|TAC|GGT|GGT|
                          olig#8 = 3'- g cca cca

                                  / 3' = olig#7
|TGC|CGT|GCT|AAG|CGT|AAC|AAC|TTT|AAA|
 acg gca cga ttc gca ttg ttg aaa ttt

|TCG|GCC|GAA|GAT|TGC|ATG|CGT|ACC|TGC|GGT|
 agc cgg ctt cta acg tac gca tgg acg cca

|GGC|GCC|GCT|GAA|
 ccg cgg cgt ctt

        |TTT|ACT|TCG|AAA|GCG|TCG|CCG|
         aaa tga agc ttt cgc agc ggc -5'
```

"Top" strand	93	
"Bottom" strand	97	
Overlap	25	(15 g/c & 10 a/t)
Net length	146	

Table 32: DNA_seq4

			g	a	a	e	g	d	d	
5'			80	81	82	83	84	85	86	
cct	cgc	cct	GGC	GCC	GCT	GAA	GGT	GAT	GAT	
spacer			Bbe I							
			Nar I							

p	a	k	a	a
87	88	89	90	91
CCG	GCC	AAA	GCG	GCC
Sfi I				

f	n	s	l	q	a	s	a	t
92	93	94	95	96	97	98	99	100
TTT	AAC	TCT	CTG	CAA	GCT	TCT	GCT	ACC
				Hind 3				

e	y	i	g	y	a	w
101	102	103	104	105	106	107
GAA	TAT	ATC	GGT	TAC	GCG	TGG
				Mlu I		

a	m	v	v	v
108	109	110	111	112
GCC	ATG	GTG	GTG	GTT
BstX I				
Nco I				

i	v	g	a	t	i	g	i
113	114	115	116	117	118	119	120
ATC	GTT	GGT	GCT	ACC	ATC	GGT	ATC

k	l	f	k	k	f	t	s	k				
121	122	123	124	125	126	127	128	129				
AAA	CTG	TTT	AAG	AAA	TTT	ACT	TCG	AAa	gcg	tcg	ggc	- 3'
						Asu II	spacer					

Table 33: DNA_synth4

```
5' |GCT|CGC|CCT|GGC|GCG|GCT|GAA|GGT|GAT|GAT|

   |CCG|GCC|AAA|GCG|GCC|

   |TTT|AAC|TCT|CTG|CAA|GCT|TCT|GCT|ACC|

              |GAA|TAT|ATC|GGT|TAC|GCG|TGG|
olig#10 = 3'- ata tag cca atg cgc acc

          / 3' = olig#9
|GCC|ATG|GTG|GTG|GTT|
 cgg tac cac cac caa

|ATC|GTT|GGT|GCT|ACC|ATC|GGT|ATC|
 tag caa cca cga tgg tag cca tag

|AAA|CTG|TTT|AAG|AAA|TTT|ACT|TCG|AAA|GCG|TCT|TGA|
 ttt gac aaa ttc ttt aaa tga agc ttt cgc aga act - 5'
```

"Top" strand	100	
"Bottom" strand	93	
Overlap	25	(14 c/g and 11 a/t)
Net length	149	

Table 34: Some interaction sets in BPTI

Res. #	Number Diff. AAs	Contents	BPTI	1	2	3	4	5
-5	2	D -32	-					
-4	2	E -32	-					
-3	5	T P F Z -29	-					
-2	10	Z3 R3 Q2 T2 H G L K E -18	-					
-1	10	D4 T2 P2 Q2 E G N K R -18	-					
1	10	R21 A2 K2 H2 P L I T G D	R					5
2	9	P20 R4 A2 H2 N E V F L	P				s	5
3	10	D15 K6 T3 R2 P2 S Y G A L	D				4	s
4	7	F19 D4 L3 Y2 I2 A2 S	F				s	5
5	1	C33	C				x	x
6	10	L11 E5 N4 K3 Q2 I2 Y2 D2 T R	L				4	
7	5	L18 E11 K2 S Q	E			s	4	
8	7	P26 H2 A2 I L G F	P			3	4	
9	9	P17 A6 V3 R2 Q L K Y F	P		s	3	4	
10	10	Y11 E7 D4 A2 N2 R2 V2 S I D	Y	s		s	4	
11	10	T17 P5 A3 R2 I S Q Y V K	T	1	s	3	4	
12	2	G32 K	G	x		x	x	
13	5	P22 R6 L3 N I	P	1		s	4	s
14	3	C31 T A	C	1		s	s	5
15	12	K15 R4 Y2 M2 L2 -2 V G A I N F	K	1	s	3	4	s
16	7	A22 G5 Q2 R K D F	A	1	s	s	s	5
17	12	R12 K5 A2 Y3 H2 S2 F2 L M T G P	R	1	2	3		s
18	6	I21 M4 F3 L2 V2 T	I	1	s	s		5
19	7	I11 P10 R6 S2 K2 L Q	I	1	2	3		s
20	5	R19 A7 S4 L2 Q	R	s	s	s		5
21	4	Y18 F13 W I	Y		2	s	s	s
22	6	F14 Y14 H2 A N S	F		s	3	4	
23	2	Y32 F	Y			s	s	
24	4	N26 K3 D3 S	N		s	3		
25	10	A12 S5 Q3 P3 W3 L2 T2 K G R	A			s	s	
26	9	K16 A6 T2 E2 S2 R2 G H V	K		s	3	4	
27	5	A18 S8 K3 L2 T2	A		2	3	4	
28	7	G13 K10 N5 Q2 R H M	G		2	s	s	
29	10	L9 Q7 K7 A2 F2 R2 M G T N	L		2	3		
30	1	C33	C		x	x	x	
31	7	Q12 E11 L4 K2 V2 Y N	Q		2	3	4	
32	11	T12 P5 K4 Q3 E2 L2 G V S R A	T		2	3	s	
33	1	F33	F	x	x	x	x	
34	11	V11 I8 T3 D2 N2 Q2 F H P R K	V	1	2	3	s	
35	2	Y31 W2	Y	s	s	s		5
36	3	G27 S5 R	G	1				
37	1	G33	G	x				x
38	3	C31 T A	C	1			s	5
39	7	R13 G9 K4 Q3 D2 P M	R	1			4	s

Table 34: continued.

Res. #	Number Diff. AAs	Contents	BPTI	1	2	3	4	5
40	2	G22 A11	A	s			s	5
41	3	N20 K11 D2	K				4	s
42	9	A11 R9 S4 G3 H2 D Q K N	R				s	5
43	2	N31 G2	N					s
44	3	N21 R11 K	N					s
45	2	F32 Y	F					s
46	8	K24 E2 S2 D H V Y R	K					5
47	2	T19 S14	S		s			5
48	9	A11 I9 E4 T2 W2 L2 R K D	A		2	s		s
49	7	E19 D6 A2 Q2 K2 T H	E		2			s
50	6	E16 D12 L2 M Q K	D		s			5
51	1	C33	C		x			x
52	7	R13 M10 L3 E3 Q2 H V	M		2			s
53	8	R21 Q3 E2 H2 C2 G K D	R		s			5
54	7	T23 A3 V2 E2 I Y K	T					5
55	1	C33	C					x
56	8	G15 V8 I3 E2 R2 A L S	G					
57	8	G19 V4 A3 P2 -2 R L N	G					
58	8	A11 -10 P3 K3 S2 Y2 R F	A					
59	9	-24 G2 Q E A Y S P R	-					
60	6	-28 Q R I G D	-					
61	3	-31 T P	-					
62	2	-32 D	-					
63	2	-32 K	-					
64	2	-32 S	-					

s indicates secondary set
x indicates in or close to surface but buried and/or highly conserved.

Table 35:
Distances from C_{beta} to
Tip of Side Group
in Angstroms

Amino Acid type	Distance
A	0.0
C (reduced)	1.8
D	2.4
E	3.5
F	4.3
G	-
H	4.0
I	2.5
K	5.1
L	2.6
M	3.8
N	2.4
P	2.4
Q	3.5
R	6.0
S	1.5
T	1.5
V	1.5
W	5.3
Y	5.7

Notes: These distances were calculated for standard model parts with all side groups fully extended.

Table 36: Distances, BPTI residue set #2

Distances in Angstroms between C_{beta}s.

Hypothetical C_{beta} was added to each Glycine.

	R17	I19	Y21	A27	G28	L29	Q31	T32	V34	A48
I19	7.7									
Y21	15.1	8.4								
A27	22.6	17.1	12.2							
G28	26.6	20.4	13.8	5.3						
L29	22.5	15.8	9.6	5.1	5.2					
Q31	16.1	10.4	6.8	6.8	10.6	6.8				
T32	11.7	5.2	6.1	12.0	15.5	10.9	5.4			
V34	5.6	6.5	11.6	17.6	21.7	18.0	11.4	8.2		
A48	18.5	11.0	5.4	12.6	13.3	8.4	8.8	8.3	15.7	
E49	22.0	14.7	8.9	16.9	16.1	12.2	13.9	13.3	19.8	5.5
M52	23.6	16.3	8.6	12.2	10.3	7.6	11.3	12.2	20.0	6.2
P9	14.0	11.3	9.0	12.2	15.4	13.3	7.9	9.2	8.7	13.9
T11	9.5	11.2	13.5	18.8	22.5	19.8	13.5	12.1	5.7	18.5
K15	7.9	14.6	20.1	27.4	31.3	27.9	21.4	18.1	10.3	24.6
A16	5.5	10.1	15.9	25.2	28.5	24.6	18.6	14.5	8.6	19.8
I18	6.1	6.0	11.2	21.3	24.4	20.2	14.7	10.4	7.0	15.0
R20	10.6	5.9	5.4	16.0	18.5	14.6	9.8	6.9	7.8	10.2
F22	15.6	10.9	5.6	10.5	12.8	10.3	6.2	8.1	10.8	10.3
N24	19.9	14.7	9.4	4.1	7.2	6.1	4.8	10.0	14.7	11.4
K26	24.4	20.1	15.2	5.4	7.7	9.3	10.1	15.3	19.0	17.0
C30	18.9	12.1	4.6	8.8	9.5	5.3	5.9	8.2	14.9	4.9
F33	10.8	7.4	7.7	12.6	16.4	13.0	6.6	5.6	5.5	12.2
Y35	8.4	7.4	9.4	18.4	21.4	17.9	12.2	9.5	5.8	14.4
S47	17.6	10.6	6.6	17.3	17.9	13.4	12.6	10.4	15.9	5.3
D50	20.0	13.6	7.2	17.2	16.8	13.5	13.5	12.9	17.6	7.6
C51	18.9	12.2	4.0	12.1	12.2	8.8	8.8	9.7	15.3	5.4
R53	25.4	18.6	11.0	17.2	15.0	13.0	15.7	16.7	22.3	9.7
R39	15.4	16.9	17.1	24.9	27.2	24.9	20.1	18.7	13.8	22.3

Table 36, continued.

Distances in Angstroms between C_{beta}s.

Hypothetical C_{beta} was added to each Glycine.

	E49	M52	P9	T11	K15	A16	I18	R20	F22	N24
M52	6.1									
P9	17.7	15.5								
T11	22.1	21.5	7.2							
K15	27.5	28.7	16.4	9.5						
A16	22.2	24.2	14.9	9.8	6.2					
I18	17.4	19.5	12.2	9.5	10.4	4.9				
R20	13.0	13.8	8.0	9.4	14.9	10.6	6.2			
F22	13.8	11.4	4.1	10.6	19.1	16.3	12.7	6.9		
N24	15.6	11.2	8.4	15.3	24.1	21.9	18.2	12.7	6.6	
K26	20.9	15.7	12.1	18.6	27.9	26.6	23.3	18.1	11.6	5.9
C30	8.7	5.6	10.6	16.6	24.1	20.2	15.7	9.8	6.8	6.9
F33	16.5	15.4	4.2	7.1	15.0	12.8	9.6	6.1	5.6	9.3
Y35	17.2	17.8	7.8	5.8	11.0	7.6	4.9	4.3	8.8	14.8
S47	4.7	9.1	15.3	18.5	23.1	17.6	12.8	9.1	12.0	15.3
D50	5.5	7.7	14.7	18.6	24.2	19.2	14.7	9.9	11.0	14.7
C51	7.1	5.4	11.0	16.4	23.5	19.2	14.6	8.7	6.9	9.6
R53	6.3	5.6	17.9	23.1	29.6	24.8	20.3	15.0	13.8	15.5
R39	23.9	24.0	13.0	9.5	12.0	11.8	12.5	12.8	14.7	20.9

	K26	C30	F33	Y35	S47	D50	C51	R53
C30	12.4							
F33	13.9	10.1						
Y35	19.5	13.5	6.4					
S47	21.0	8.8	13.5	13.2				
D50	20.1	8.6	14.3	13.7	5.0			
C51	15.0	3.7	10.9	12.5	6.9	5.2		
R53	19.9	9.9	18.2	18.8	9.4	5.8	7.4	
R39	24.3	20.6	14.4	9.6	20.4	19.0	18.8	23.4

Table 37: vgDNA to vary BPTI set #2.1

```
                                  | g | p | c | k | a | X |
                                  | 35| 36| 37| 38| 39| 40|
5'-|CAC|CCT|GGG|CCC|TGC|AAA|GCG|qfk|                          206
   |spacer | Apa I |

      +
| i | X | r | y | f | y | n | a | k |
| 41| 42| 43| 44| 45| 46| 47| 48| 49|
|ATC|qfk|CGT|TAT|TTC|TAC|AAC|GCT|AAA|                          235

                                        /  3' = olig#27  72 nts
      +   !   +                         |   +
| X | g | X | c | q | t | f | X | y | g | q |
| 50| 51| 52| 53| 54| 55| 56| 57| 58| 59| 60|
|qfk|GGt|qfk|TGC|CAG|ACC|TTc|qfk|TAC|GGT|GGT|                  268
olig#28= 3'- acg gtc tgg aag **m atg cca cca
78 nts

Overlap = 12 (7 CG, 5 AT)

| c | r | a | k | r | n | n | f | k |
| 61| 62| 63| 64| 65| 66| 67| 68| 69|
|TGC|CGT|GCT|AAG|CGT|AAC|AAC|TTT|AAA|                          295
 acg gca cga ttc gca ttg ttg aaa ttt
       | Esp I  |

      +
| s | X | e | d | c | m |
| 70| 71| 72| 73| 74| 75|
|TCT|qfk|GAG|GAT|TGC|ATG|C                                    322
 agc **m ctc cta acg tac gca ccc acc  -5'
                  | Sph I|  spacer   |
```

k = equal parts of T and G; m = equal parts of C and A;
q = (.26 T, .18 C, .26 A, and .30 G);
f = (.22 T, .16 C, .40 A, and .22 G);
* = complement of symbol above

Residue 40 42 50 52 57 71
Possibilities 21 x 21 x 21 x 21 x 21 x 21 = 8.6×10^7
Abundance x 10:
of PPBD .768 .271 .459 .671 .600 .459
Produce = 1.77×10^{-3}

Parent = $1/(5.5 \times 10^7)$ least favored = $1/(4.2 \times 10^9)$
Least favored one-amino-acid substitution from PPBD present at 1 in 1.6×10^7

Table 38: Result of varying set#2 of BPTI 2.1

								l	e	
								29	30	
								CTC	GAG	178
								Ava I		
								Xho I		

p	p	y	t	g	p	c	k	a	D	
31	32	33	34	35	36	37	38	39	40	
CCG	CCA	TAT	ACT	GGG	CCC	TGC	AAA	GCG	GAT	208
PflM I										
				Apa I						
				Dra II						
				Pss I						

i	Q	r	y	f	y	n	a	k	
41	42	43	44	45	46	47	48	49	
ATC	CAG	CGT	TAT	TTC	TAC	AAC	GCT	AAA	235

E	g	L	c	q	t	f	S	y	g	g	
50	51	52	53	54	55	56	57	58	59	60	
GAG	GGC	CTG	TGC	CAG	ACC	TTT	TCG	TAC	GGT	GGT	268

c	r	a	k	r	n	n	f	k	
61	62	63	64	65	66	67	68	69	
TGC	CGT	GCT	AAG	CGT	AAC	AAC	TTT	AAA	295
	Esp I								

s	W	e	d	c	m	r	t	c	g	
70	71	72	73	74	75	76	77	78	79	
TCG	TGG	GAA	GAT	TGC	ATG	CGT	ACC	TGC	GGT	325
				Sph II						

g	a	
80	81	
GGC	GCC	
Bpe I		
Nar I		

Table 39: vgDNA to vary set#2 BPTI 2.2

```
                                        +
                                | g | p | c | X | a | D |
                                | 35| 36| 37| 38| 39| 40|
5' - cg gca cgc|GGG|CCC|TGC|mrA|GCG|GAT|                    208
     | spacer  | Apa I |

  +       +   +
| X | Q | X | X | f | y | n | a | k |
| 41| 42| 43| 44| 45| 46| 47| 48| 49|
|rwA|CAG|rvk|T-T|TTC|TAC|AAC|GCT|AAA|                    235

      +                   +   +
| E | X | L | c | X | X | f | S | y | g | g |
| 50| 51| 52| 53| 54| 55| 56| 57| 58| 59| 60|
|GAG|qfk|CTG|TGC|qfk|qfk|TTT|TCG|TAC|GGT|GGT|            268
                91 nts olig#30 3'- g cca cca

  Overlap = 15 (11 CG, 4 AT)

            /- 3' olig#29  94 nts
| c | r | a | k | r | n | n | f | k |
| 61| 62| 63| 64| 65| 66| 67| 68| 69|
|TGC|CGT|GCT|AAG|CGT|AAC|AAC|TTT|AAA|                    295
 acg gca cga ttc gca ttg ttg aaa ttt
       | Esp I |
          +
| s | W | X | d | c | m |
| 70| 71| 72| 73| 74| 75|
|TCG|TGG|qfk|GAT|TGC|ATG|C
 agc acc **m cta acg tac gcg acc tgc -5'
                  | Sph I|  spacer |
```

k = equal parts of T and G; v = equal parts of C, A, and G;
m = equal parts of C and A; r = equal parts of A and G;
w = equal parts of A and T;
q = (.26 T, .18 C, .26 A, and .30 G);
f = (.22 T, .16 C, .40 A, and .22 G);
* = complement of symbol above

Residue 38 41 43 44 51 54 55 72
Possibilities 4 x 4 x 9 x 2 x 21 x 21 x 21 x 21
$= 6.2 \times 10^7$

Abundance x 10 2.5 2.5 .833 5. .663 .397 .437 .602
Product = 2.3×10^{-8}

Parent = $1/(4.4 \times 10^7)$ least favored = $1/(1.25 \times 10^9)$
Least favored one-amino-acid substitution from PPBO present at 1 in 1.2×10^7

Table 40: Result of varying set#2 of BPTI 2.2

						l	c			
						29	30			
						CTC	GAG			178
						Xho I				
p	p	y	t	q	p	c	E	a	D	
31	32	33	34	35	36	37	38	39	40	
CCG	CCA	TAT	ACT	GGG	CCC	TGC	GAG	GCG	GAT	208
PflM I					Apa I					
V	Q	N	F	f	y	n	a	k		
41	42	43	44	45	46	47	48	49		
GTT	CAG	AAT	TTT	TTC	TAC	AAC	GCT	AAA		235
E	F	L	c	S	A	f	S	y	g	g
50	51	52	53	54	55	56	57	58	59	60
GAG	TTT	CTG	TGC	TCT	GCT	TTT	TCG	TAC	GGT	GGT	268
c	r	a	k	r	n	n	f	k		
61	62	63	64	65	66	67	68	69		
TGC	CGT	GCT	AAG	CGT	AAC	AAC	TTT	AAA		295
		Esp I								
s	W	Q	d	c	m	r	t	c	g	
70	71	72	73	74	75	76	77	78	79	
TCG	TGG	CAG	GAT	TGC	ATG	CGT	ACC	TGC	GGT	325
				Sph I						
g	a									
80	81									
GGC	GCC									
Rbe I										
Nar I										

Table 41: vg DNA set#2 of BPTI 2.3

```
                                                    | l  | e  |
                                                    | 29 | 30 |
                                5'- cg agc ctg      |CTC |GAG |                178
                                    | spacer        | Xho I |

                                                                  +
| p  | X  | y  | X  | g  | p  | c  | E  | a  | X  |
| 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
|CCG |vmg |TAT |vmg |GGG |CCC |TGC |GAG |GCG |qfk |                        208

                                  +
| V  | Q  | N  | X  | f  | y  | n  | a  | k  |
| 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |
|GTT |CAG |AAT |Tdk |TTC |TAC |AAC |GCG |AAG | -3' olig#33  71 nts
67 nts olig#34 3'- g atg ttg cgg ttc

  Overlap = 13 (7 CG, 6 AT)

  +          +          +          +
| X  | F  | X  | c  | S  | X  | f  | X  | y  | g  | g  |
| 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 |
|vAG |TTT |nTk |TGC |TCT |qfk |TTT |qfk |TAC |GGT |GGT |                    268
 btc aaa nam acg aga **m aaa **m atg cca cca

| c  | r  | a  | k  |
| 61 | 62 | 63 | 64 |
|TGC |CGT |GCT |AAG |C
 acg gca cga ttc gcg acc ggc
       | Esp I  | spacer  |
```

k = equal parts of T and G; m = equal parts of C and A;
w = equal parts of A and T; n = equal parts of A,C,G,T;
d = equal parts A,G,T; v = equal parts A,C,G;
q = (.26 T, .18 C, .26 A, and .30 G);
f = (.22 T, .16 C, .40 A, and .22 G);
* = complement of symbol above

Residue Possibilities: 32 (6 x) 34 (6 x) 40 (21 x) 44 (6 x) 50 (3 x) 52 (5 x) 55 (21 x) 57 (21 = 3 x 10^7)

Abundance x 10 of PPBD: 10/6 10/6 .545 10/6 10/3 30/8 .459 .701
product = 1.01 x 10^{-7}

parent = $1/(1 \times 10^7)$ least favored = $1/(4 \times 10^8)$
Least favored one-amino-acid substitution from PPBD present at 1 in 3 x 10^7

Table 42: Result of varying set#2 of BPTI 2.3

								l	e	
								29	30	
								CTC	GAG	178
								Ava I		
								Xho I		

p	E	y	Q	g	p	c	r	a	A	
31	32	33	34	35	36	37	38	39	40	
CCG	GAG	TAT	CAG	GGG	CCC	TGC	GAG	GCG	GCT	208
				Apa I						

V	Q	N	W	f	y	n	a	k	
41	42	43	44	45	46	47	48	49	
GTT	CAG	AAT	TGG	TTC	TAC	AAC	GCT	AAA	235

Q	F	M	c	S	L	f	H	y	g	g	
50	51	52	53	54	55	56	57	58	59	60	
CAG	TTT	ATG	TGC	TCT	CTT	TTT	CAT	TAC	GGT	GGT	268

c	r	a	k	r	n	n	f	k	
61	62	63	64	65	66	67	68	69	
TGC	CGT	GCT	AAG	CGT	AAC	AAC	TTT	AAA	295
	Esp I								

s	W	Q	d	c	m	r	t	c	g	
70	71	72	73	74	75	76	77	78	79	
TCG	TCG	CAG	GAT	TGC	ATG	CGT	ACC	TGC	GGT	325
				Sph I						

g	a	
80	81	
GGC	GCC	
Bbe I		
Nar I		

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CLAIMS

1. A method of obtaining a protein that binds a predetermined target that comprises:

a) preparing a variegated population of replicable genetic packages, each package including a nucleic acid construct coding on expression for an outer-surface-displayed potential binding protein comprising (i) a structural signal directing the display of the protein on the outer surface of the package and (ii) a potential binding domain for binding said target, where a plurality of different potential binding domains are displayed by said population,

b) causing the expression of said proteins and the display of said proteins on the outer surface of such packages,

c) contacting the packages with target material so that the potential binding domains of the proteins and the target material may interact, and separating packages bearing a binding domain that binds target material from packages that do not so bind, and

d, recovering and replicating at least one package bearing a successful binding domain.

2. The method of claim 1 wherein the population of replicable genetic packages of step (a) is obtained by:

i) preparing a variegated population of DNA

CLAIMS

1. A method of obtaining a protein that binds a predetermined target that comprises:

a) preparing a variegated population of replicable genetic packages, each package including a nucleic acid construct coding on expression for an outer-surface-displayed potential binding protein comprising (i) a structural signal directing the display of the protein on the outer surface of the package and (ii) a potential binding domain for binding said target, where a plurality of different potential binding domains are displayed by said population,

b) causing the expression of said proteins and the display of said proteins on the outer surface of such packages,

c) contacting the packages with target material so that the potential binding domains of the proteins and the target material may interact, and separating packages bearing a binding domain that binds target material from packages that do not so bind, and

d) recovering and replicating at least one package bearing a successful binding domain.

2. The method of claim 1 wherein the population of replicable genetic packages of step (a) is obtained by:

i) preparing a variegated population of DNA

certain predetermined degree of affinity for target material, and the required degree of affinity is increased for each new variegated population.

7. The method of claim 1 wherein the displayable potential binding protein is a chimeric protein.

8. The method of claim 7 wherein said signal is provided by a segment of said chimeric protein which is essentially identical in amino acid sequence with at least a functional portion of a natural outer surface protein encoded by said genetic package or a cell naturally infected by said genetic package, said portion directing the transport of said chimeric protein to the outer surface of the genetic package.

9. The method of claim 2 wherein the second sequence is obtained by operably linking a DNA sequence encoding a potential outer surface transport signal to a DNA sequence expressing a protein that confers a selectable phenotype to obtain a test construct, introducing the test constructs into suitable hosts, causing expression of said DNA construct, selecting genetic packages that display the protein that confers the selectable phenotype on their outer surface, and choosing as said second sequence the DNA sequence encoding the potential outer surface transport signal of one of such selected genetic packages; wherein the potential outer surface transport signals encoded by the individual test constructs are non-identical.

certain predetermined degree of affinity for target material, and the required degree of affinity is increased for each new variegated population.

7. The method of claim 1 wherein the displayable potential binding protein is a chimeric protein.

8. The method of claim 7 wherein said signal is provided by a segment of said chimeric protein which is essentially identical in amino acid sequence with at least a functional portion of a natural outer surface protein encoded by said genetic package or a cell naturally infected by said genetic package, said portion directing the transport of said chimeric protein to the outer surface of the genetic package.

9. The method of claim 2 wherein the second sequence is obtained by operably linking a DNA sequence encoding a potential outer surface transport signal to a DNA sequence expressing a protein that confers a selectable phenotype to obtain a test construct, introducing the test constructs into suitable hosts, causing expression of said DNA construct, selecting genetic packages that display the protein that confers the selectable phenotype on their outer surface, and choosing as said second sequence the DNA sequence encoding the potential outer surface transport signal of one of such selected genetic packages; wherein the potential outer surface transport signals encoded by the individual test constructs are non-identical.

17. The method of claim 3 in which the binding domain of the known protein has a known sequence of amino acids, and the identity and spatial relationship of the amino acids forming a surface of said domain is known.

18. The method of claim 3, said target material comprising one or more discrete molecules, said parental potential binding domain being characterized as a sequence of amino acids, further comprising identifying an interaction set of amino acids which are on the surface of the parental potential binding domain and which can all simultaneously touch a single molecule of the target material, and obtaining potential binding domains by substituting a different amino acid for one or more of the amino acids in said interaction set.

19. The method of claim 3 wherein the level of variegation of the population is chosen such that the packages displaying potential binding domains obtained by single amino acid substitutions in the amino acid sequence of the parental potential binding domain are present in detectable amounts.

20. The method of claim 3 wherein the amino acid substitutions to be made are chosen after consideration of the 3D structure of the parental potential binding domain.

21. The method of claim 15 wherein the amino acid substitutions to be made are for amino acids of the chosen domain of the known protein which are known to be alterable without reducing the melting

17. The method of claim 3 in which the binding domain of the known protein has a known sequence of amino acids, and the identity and spatial relationship of the amino acids forming a surface of said domain is known.

18. The method of claim 3, said target material comprising one or more discrete molecules, said parental potential binding domain being characterized as a sequence of amino acids, further comprising identifying an interaction set of amino acids which are on the surface of the parental potential binding domain and which can all simultaneously touch a single molecule of the target material, and obtaining potential binding domains by substituting a different amino acid for one or more of the amino acids in said interaction set.

19. The method of claim 3 wherein the level of variegation of the population is chosen such that the packages displaying potential binding domains obtained by single amino acid substitutions in the amino acid sequence of the parental potential binding domain are present in detectable amounts.

20. The method of claim 3 wherein the amino acid substitutions to be made are chosen after consideration of the 3D structure of the parental potential binding domain.

21. The method of claim 15 wherein the amino acid substitutions to be made are for amino acids of the chosen domain of the known protein which are known to be alterable without reducing the melting

affinity separated and retain viability.

30. The method of claim 3 in which the initially chosen parental potential binding protein has at least one stable binding domain and said domain has a melting point of at least 60°C and is stable over a pH range of at least 3.0-8.0.

31. The method of claim 15 wherein the known binding protein is an enzyme, the activity of which has a deleterious effect on the replicable genetic package, the host of the replicable genetic package, or the target, wherein the majority of the nucleic acid constructs code on expression or an analogue of the known binding protein that does not have such enzymatic activity.

32. The method of claim 1 wherein the target contains ionizable groups and the pH of the solutions of the intended use and the pH of the affinity separations are chosen so that both the potential binding protein and the target remain stable.

33. The method of claim 1 wherein the target contains ionizable groups, further comprising providing counter ions in affinity separations and the solutions of the intended use to reduce electrostatic repulsion between the potential binding protein and the target.

34. The method of claim 1 wherein the initial potential binding domain is picked so that, under the conditions of intended use of the desired binding protein and under the conditions of affinity separation, that the potential binding

affinity separated and retain viability.

30. The method of claim 3 in which the initially chosen parental potential binding protein has at least one stable binding domain and said domain has a melting point of at least 60°C and is stable over a pH range of at least 3.0-8.0.

31. The method of claim 15 wherein the known binding protein is an enzyme, the activity of which has a deleterious effect on the replicable genetic package, the host of the replicable genetic package, or the target, wherein the majority of the nucleic acid constructs code on expression or an analogue of the known binding protein that does not have such enzymatic activity.

32. The method of claim 1 wherein the target contains ionizable groups and the pH of the solutions of the intended use and the pH of the affinity separations are chosen so that both the potential binding protein and the target remain stable.

33. The method of claim 1 wherein the target contains ionizable groups, further comprising providing counter ions in affinity separations and the solutions of the intended use to reduce electrostatic repulsion between the potential binding protein and the target.

34. The method of claim 1 wherein the initial potential binding domain is picked so that, under the conditions of intended use of the desired binding protein and under the conditions of affinity separation, that the potential binding

thereof embodying an outer surface transport signal.

45. The method of claim 42 wherein the signal is provided by the gene III protein of M13 or a segment thereof embodying an outer surface transport signal.

46. The method of claim 3 wherein the initially chosen parental potential binding domain is at least 50% homologous with the binding domain of bovine pancreatic trypsin inhibitor, having the residues C5, G12, C30, F33, G37, C51 and C55.

47. The method of claim 46 further specifying that: a) residue 21 contains one of the amino acids Y, F, W, or I; b) residue 23 contains one of the amino acids Y or F; c) residue 35 contains one of the residues Y, F, or W; d) residue 40 contains one of the amino acids G or A; e) residue 45 contains either F or Y.

48. The method of claim 47 wherein the residues to be varied are chosen from among residues 17, 19, 21, 27, 28, 29, 31, 32, 34, 48, 49, and 52.

49. The method of claim 48 wherein the additional residues 9, 11, 15, 16, 18, 20, 22, 24, 26, 35, 47, and 53 are allowed to vary.

50. The method of claim 47 wherein the residues to vary are picked from one of the interaction sets identified in table 34.

51. The method of claim 2 wherein the distribution of

thereof embodying an outer surface transport signal.

45. The method of claim 42 wherein the signal is provided by the gene III protein of M13 or a segment thereof embodying an outer surface transport signal.

46. The method of claim 3 wherein the initially chosen parental potential binding domain is at least 50% homologous with the binding domain of bovine pancreatic trypsin inhibitor, having the residues C5, G12, C30, F33, G37, C51 and C55.

47. The method of claim 46 further specifying that: a) residue 21 contains one of the amino acids Y, F, W, or I; b) residue 23 contains one of the amino acids Y or F; c) residue 35 contains one of the residues Y, F, or W; d) residue 40 contains one of the amino acids G or A; e) residue 45 contains either F or Y.

48. The method of claim 47 wherein the residues to be varied are chosen from among residues 17, 19, 21, 27, 28, 29, 31, 32, 34, 48, 49, and 52.

49. The method of claim 48 wherein the additional residues 9, 11, 15, 16, 18, 20, 22, 24, 26, 35, 47, and 53 are allowed to vary.

50. The method of claim 47 wherein the residues to vary are picked from one of the interaction sets identified in table 34.

51. The method of claim 2 wherein the distribution of

insensitive to UV, tolerant of desiccation, and resistant to a pH of 2.0 to 10.0.

58. The method of claim 1 wherein the genetic packages may be frozen and later revived.

59. The method of claim 1 wherein the genetic package is a cell with a doubling time of 20-40 minutes.

60. The method of claim 1 wherein the genetic package is a virus with a burst size of at least 100/infected cell.

61. The method of claim 1 wherein the genetic packages are harvested by centrifugation without loss of viability.

62. The method of claim 3 wherein the initially chosen parental potential binding domain is selected from the group consisting of (a) binding domains of bovine pancreatic trypsin inhibitor, crambin, ovomucoid, T4 lysozyme, hen egg white lysozyme, ribonuclease, and azurin, and (b) domains at least 50% homologous with any of the foregoing domains and which have a melting point of at least 60°C.

63. The method of claim 36 wherein the outer surface transport signal is provided by the lamB protein or a segment thereof embodying an outer surface transport signal.

64. The method of claim 38 wherein the outer surface transport signal is provided by the cotA, cotB, cotC or cotD protein or a segment thereof embodying an outer surface transport signal.

insensitive to UV, tolerant of desiccation, and resistant to a pH of 2.0 to 10.0.

58. The method of claim 1 wherein the genetic packages may be frozen and later revived.

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63. The method of claim 36 wherein the outer surface transport signal is provided by the lamB protein or a segment thereof embodying an outer surface transport signal.

64. The method of claim 38 wherein the outer surface transport signal is provided by the cotA, cotB, cotC or cotD protein or a segment thereof embodying an outer surface transport signal.

is further chosen to yield the largest value for the quantity ((1.-abundance(stop codons)) times (abundance of the least abundant amino acid)/(abundance of the most abundant amino acid)).

72. The protein of claim 66, wherein the protein comprises a first foreign domain recognizing a first target material and a second foreign domain recognizing a second target material.

is further chosen to yield the largest value for the quantity ((1.-abundance(stop codons)) times (abundance of the least abundant amino acid)/(abundance of the most abundant amino acid)).

72. The protein of claim 66, wherein the protein comprises a first foreign domain recognizing a first target material and a second foreign domain recognizing a second target material.

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

GENERATION AND SELECTION OF NOVEL BINDING PROTEINS

the specification of which (check one)

☐ is attached hereto.

☒ was filed on September 7, 1995
as Application Serial No. 07/240,160
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed Yes	Priority Claimed No
(Number)	(Country)	(Day/Month/Year Filed)	☐	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐	☐

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)

(Continued on Page 2)

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

GENERATION AND SELECTION OF NOVEL BINDING PROTEINS

the specification of which (check one) ☐ is attached hereto.

☒ was filed on September 2, 1998
as Application Serial No. 07/240,140
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)

(Continued on Page 2)

Applicant or Patentee: Robert C. Ladner et al. Attorney's Docket No. PEC-XBP-US
Serial or Patent No.: 07/240,160
Filed or Issued: September 7, 1988
For: GENERATION AND SELECTION OF NOVEL BINDING PROTEINS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[X] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Protein Engineering Corp.
ADDRESS OF CONCERN: 3277 Green Valley Road, Chesterville, MD 21720

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled GENERATION AND SELECTION OF NOVEL BINDING PROTEINS by inventor(s) Robert C. Ladner and Sonia K. Cwirla described in

[ ] the specification filed herewith
[X] application serial no. 07/240,160, filed September 7, 1988.
[ ] patent no. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME _____
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Dr Robert Charles LADNER
TITLE OF PERSON OTHER THAN OWNER: President
ADDRESS OF PERSON SIGNING: 3277 Green Valley Road, Chesterville, MD 21720
SIGNATURE _____ DATE _____

Applicant or Patentee: Robert C. Ladner et al. Attorney's Docket No. [illegible]-US
Serial or Patent No. [illegible]
Filed or Issued: September 7, 1988
For: GENERATION AND SELECTION OF [illegible]

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[X] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Protein Engineering Corp.
ADDRESS OF CONCERN: 3827 Green Valley Road, Evansville, WI 53536

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled GENERATION AND SELECTION OF NOVEL BINDING PROTEINS by inventor(s) Robert C. Ladner and Sonia A. Gutekunst described in:

[ ] the specification filed herewith
[X] application serial no. 07/240,160, filed September 7, 1988.
[ ] patent no. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME _____
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Dr Robert Charles LADNER
TITLE OF PERSON OTHER THAN OWNER: [illegible]
ADDRESS OF PERSON SIGNING: [illegible]
SIGNATURE _____ DATE _____

Figure 3: Process of the Present Invention

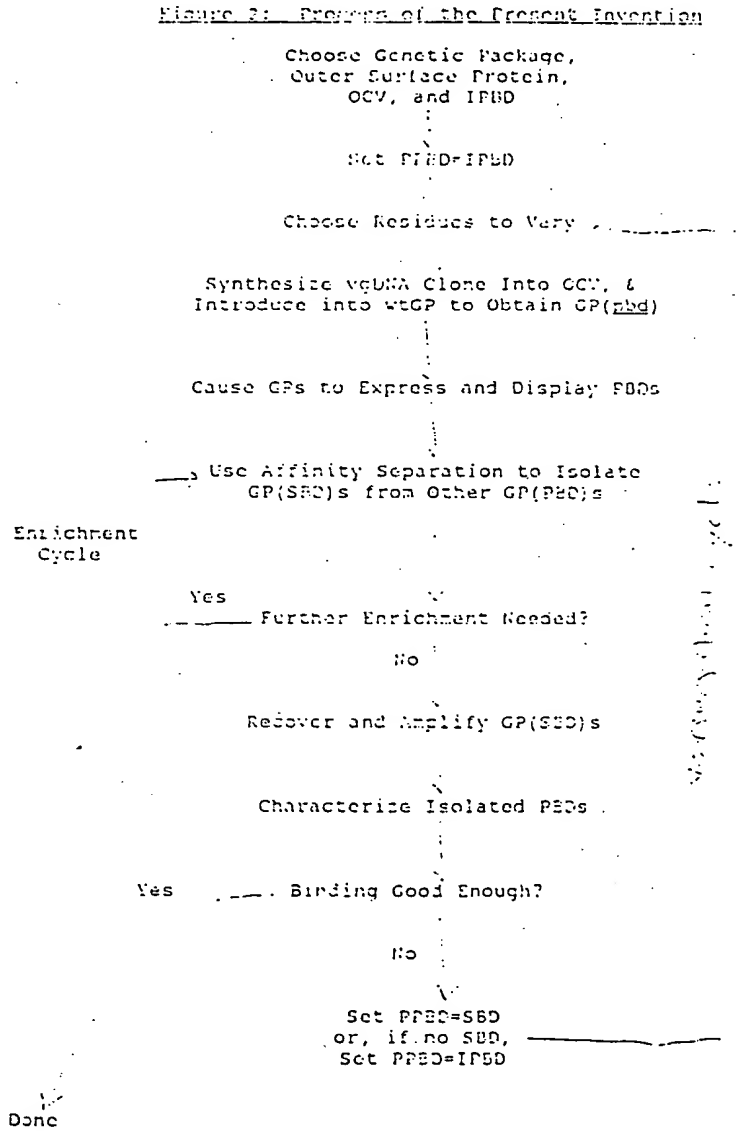
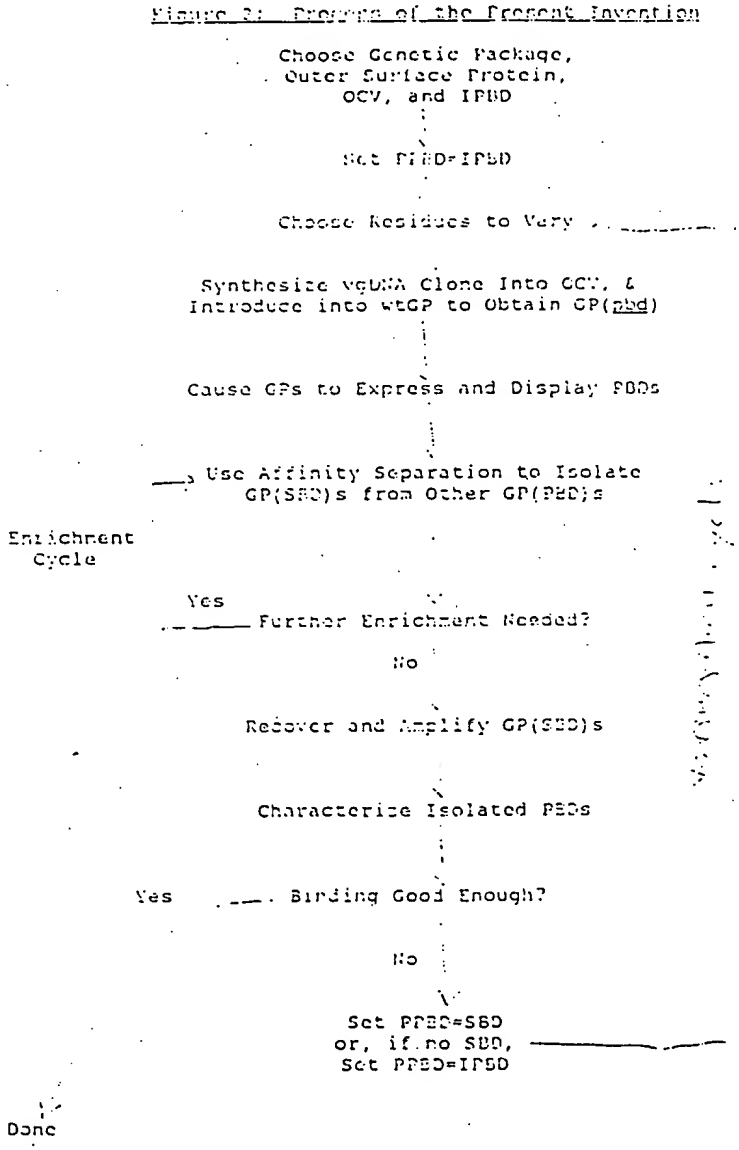
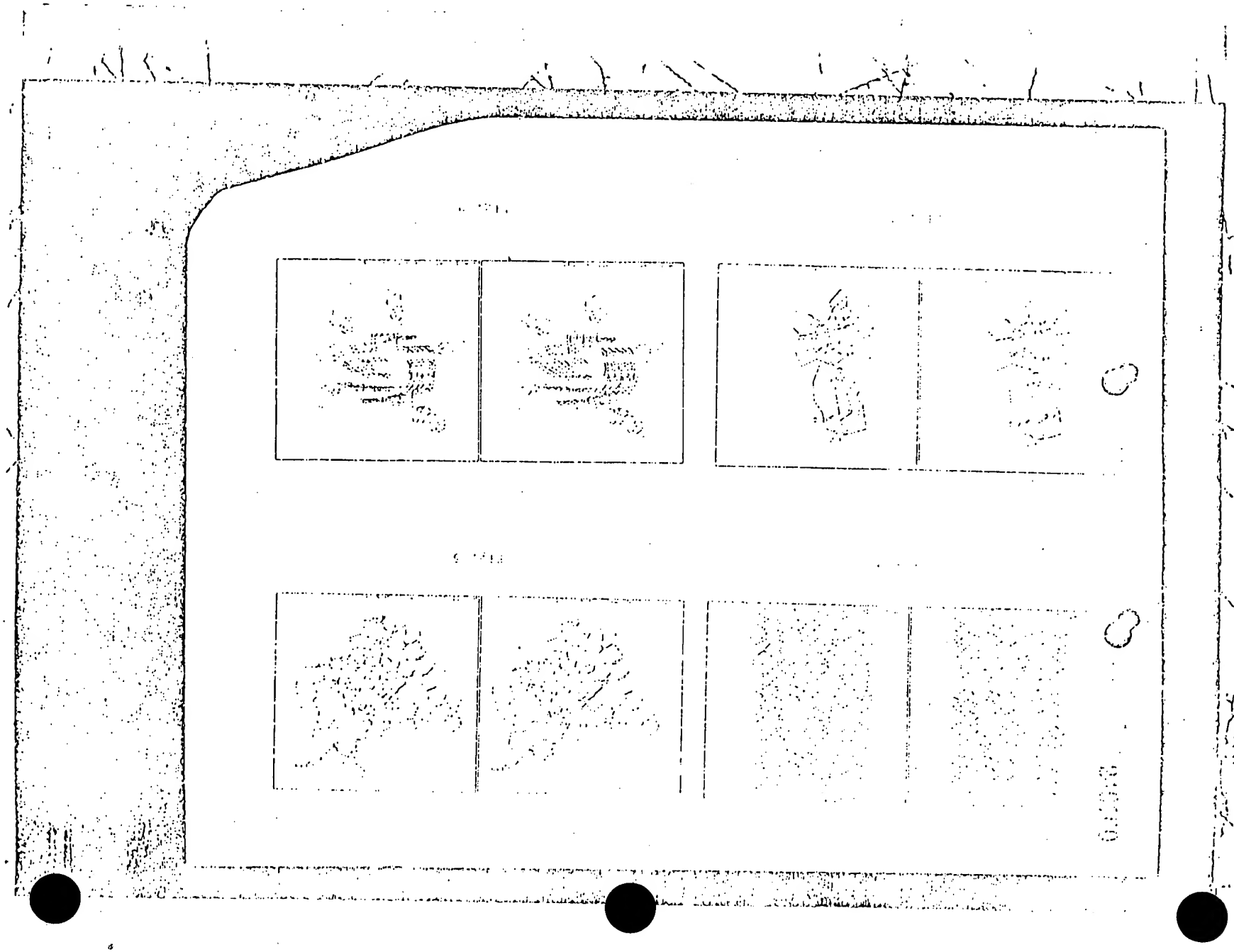


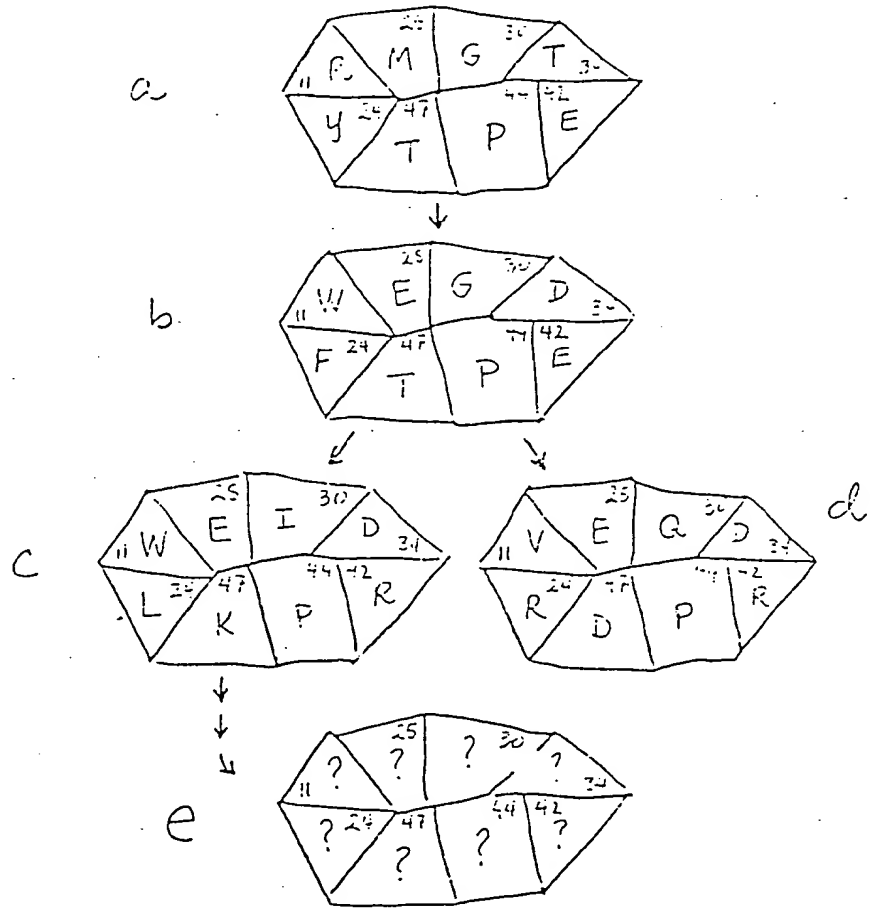
Figure 3: Process of the Present Invention





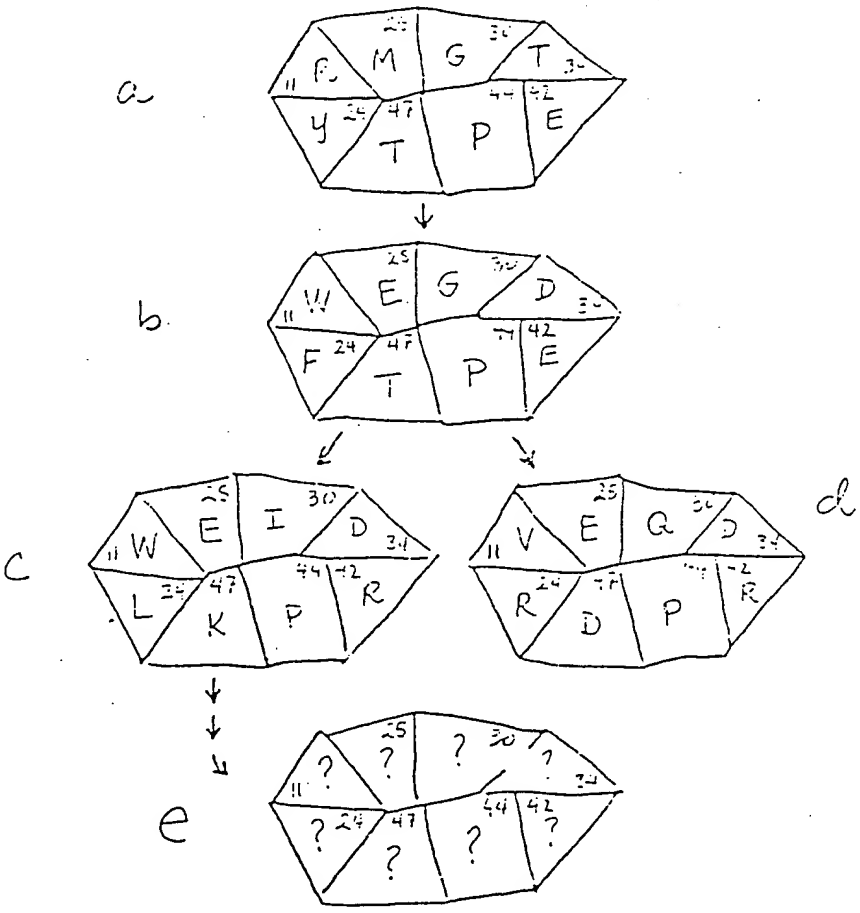
ENGINEER
Concord Engineering Corporation
1 A Concord Avenue
Cambridge, MA 02138
617 868 0 867

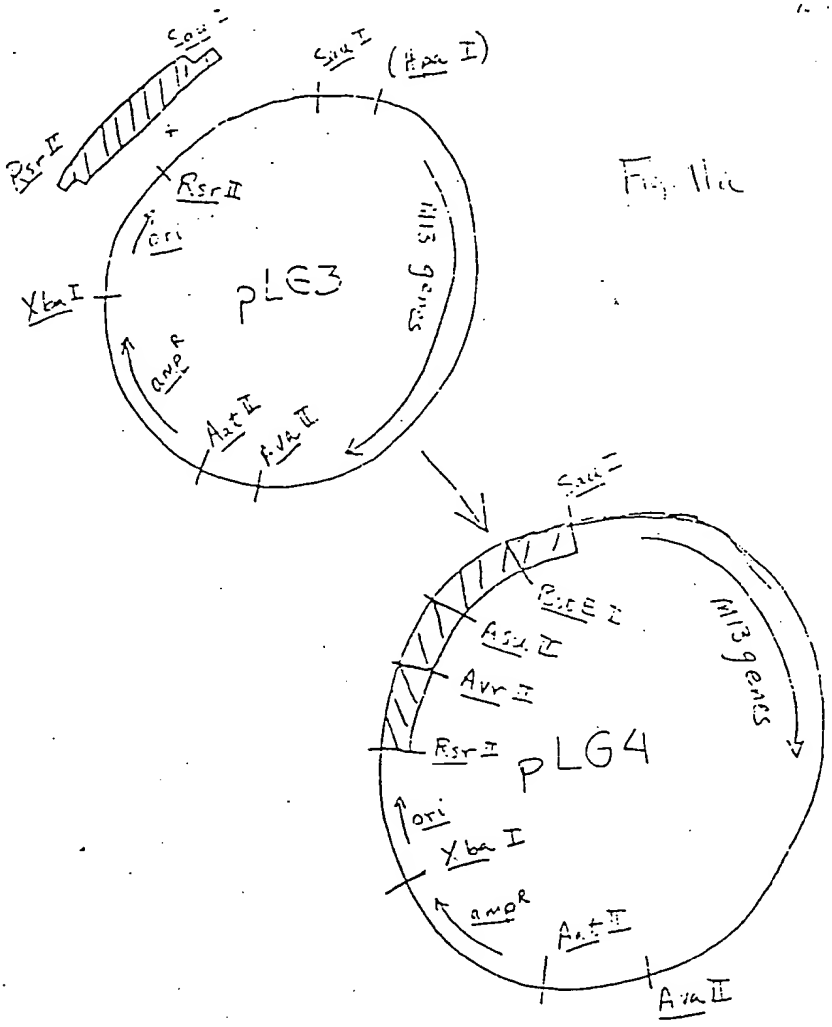
Figure 6: Binding Surface



ENGINEERING
Engineering Corporation
1 A Concord Avenue
Cambridge, MA 02138
617 868 0 697

Figure 6: Binding Surface




Sac I
Sma I (Hpa I)
Rsr II
Rsr II
f1 ori
M13 genes
Xba I
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amp R
Ava II
Ava II
Sac I
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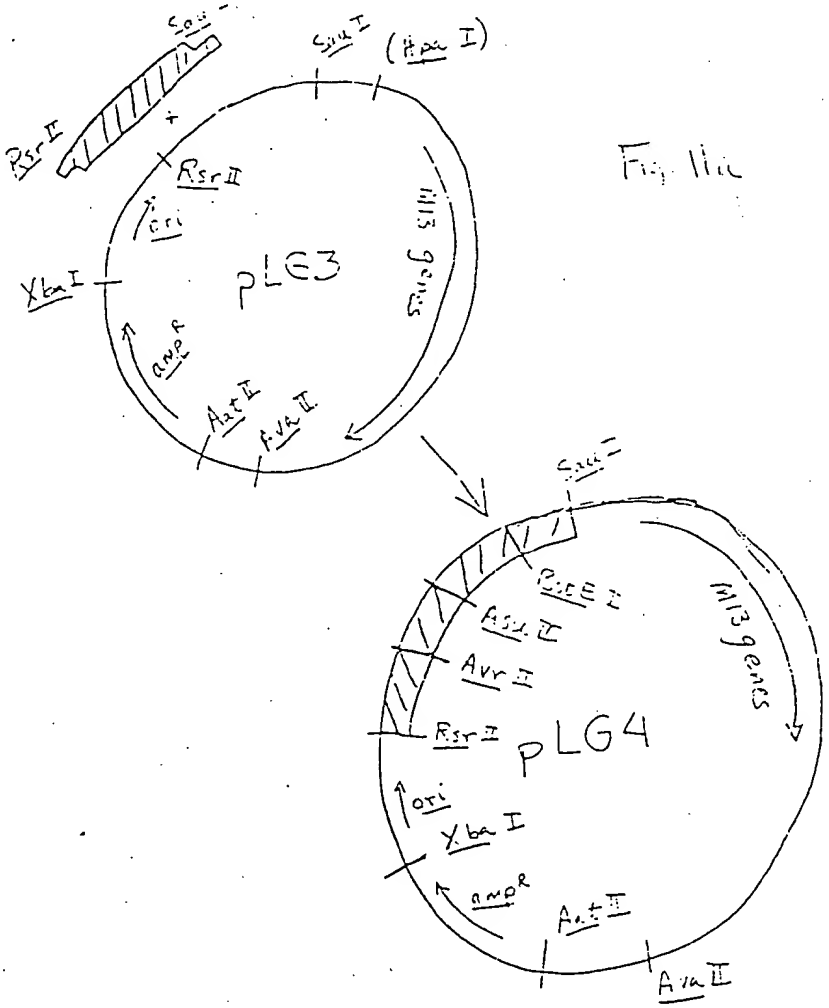
Ava II
Ava II

Fig. 11c


Sac I
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(Hpa I)
Rsr II
Rsr II
ori
pLG3
M13 gene
Xba I
amp R
Apa I
Pvu II
Sac I
Sma I
Pst I
Ava II
Avr II
Rsr II
pLG4
M13 genes
ori
Xba I
amp R
Apa II
Ava II

Fig. 11c

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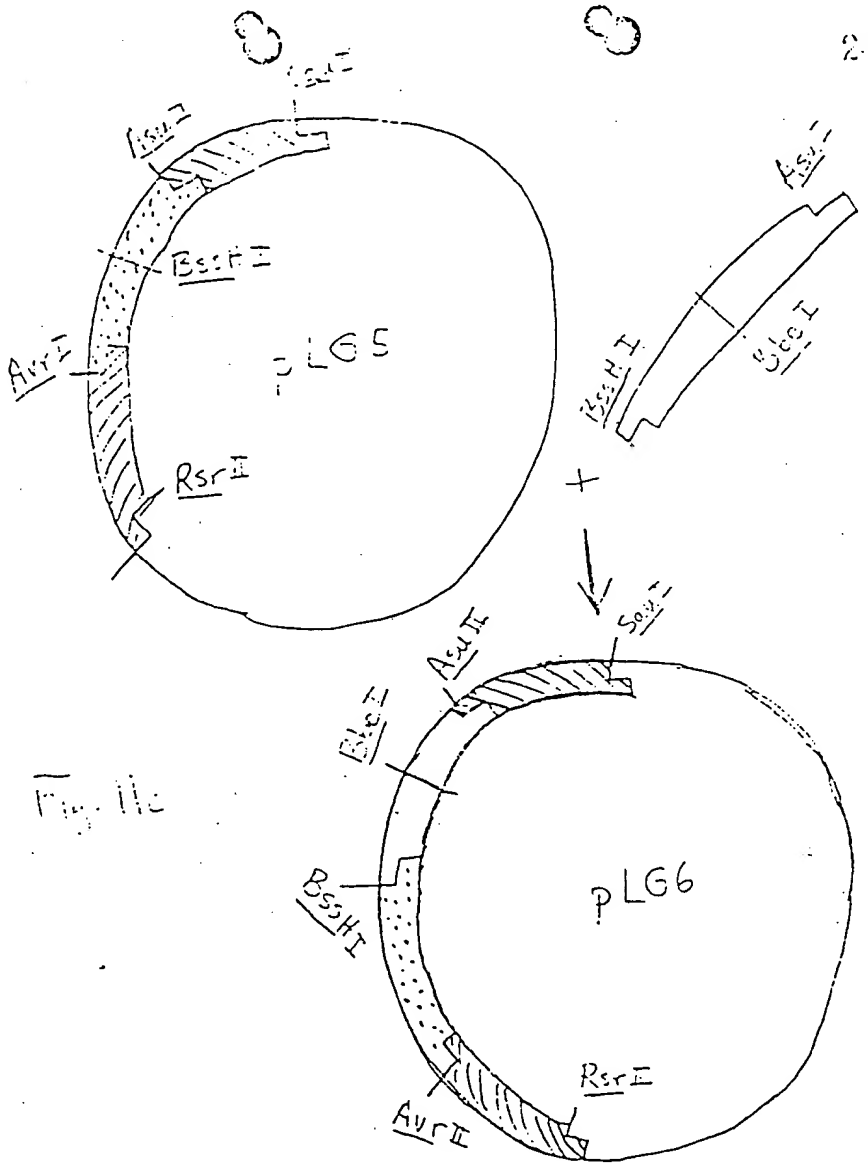
pLG5
Bsr FI
Avr II
Rsr II
Asc I
Bsa HI
Sac I
+
Sac I
Acc II
Bsa I
Bss HI
pLG6
Rsr II
Avr II

Fig. 11c

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